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(54) Title: METHODS FOR IDENTIFYING SMALL MOLECULES THAT BIND SPECIFIC RNA STRUCTURAL MOTIFS

(57) Abstract: The present invention relates to a method for screening and identifying test compounds that bind to a preselected target ribonucleic acid ("RNA"). Direct, non-competitive binding assays are advantageously used to screen libraries of compounds for those that selectively bind to a preselected target RNA. Binding of target RNA molecules to a particular test compound is detected using any physical method that measures the altered physical property of the target RNA bound to a test compound. The structure of the test compound attached to the labeled RNA is also determined. The methods used will depend, in part, on the nature of the library screened. The methods of the present invention provide a simple, sensitive assay for high-throughput screening of libraries of compounds to identify pharmaceutical leads.

METHODS FOR IDENTIFYING SMALL MOLECULES THAT BIND SPECIFIC RNA STRUCTURAL MOTIFS

This application claims the benefit of U.S. Provisional Application No. 60/282,965, filed April 11, 2001, which is incorporated herein by reference in its entirety.

1. INTRODUCTION

The present invention relates to a method for screening and identifying test compounds that bind to a preselected target ribonucleic acid ("RNA"). Direct, non-competitive binding assays are advantageously used to screen libraries of compounds for those that selectively bind to a preselected target RNA. Binding of target RNA molecules to a particular test compound is detected using any physical method that measures the altered physical property of the target RNA bound to a test compound. The methods of the present invention provide a simple, sensitive assay for high-throughput screening of libraries of compounds to identify pharmaceutical leads.

2. BACKGROUND OF THE INVENTION

Protein-nucleic acid interactions are involved in many cellular functions, including transcription, RNA splicing, mRNA decay, and mRNA translation. Readily accessible synthetic molecules that can bind with high affinity to specific sequences of single- or double-stranded nucleic acids have the potential to interfere with these interactions in a controllable way, making them attractive tools for molecular biology and medicine. Successful approaches for blocking function of target nucleic acids include using duplex-forming antisense oligonucleotides (Miller, 1996, Progress in Nucl. Acid Res. & Mol. Biol. 52:261-291; Ojwang & Rando, 1999, Achieving antisense inhibition by oligodeoxynucleotides containing N_7 modified 2′-deoxyguanosine using tumor necrosis factor receptor type 1, METHODS: A Companion to Methods in Enzymology 18:244-251) and peptide nucleic acids ("PNA") (Nielsen, 1999, Current Opinion in Biotechnology 10:71-75), which bind to nucleic acids via Watson-Crick base-pairing. Triplex-forming anti-gene oligonucleotides can also be designed (Ping *et al.*, 1997, RNA 3:850-860; Aggarwal *et al.*, 1996, Cancer Res. 56:5156-5164; U.S. Patent No. 5,650,316), as well as pyrrole-imidazole polyamide oligomers (Gottesfeld *et al.*, 1997, Nature 387:202-205; White *et al.*, 1998, Nature 391:468-471), which are specific for the major and minor grooves of a double helix, respectively.

In addition to synthetic nucleic acids (*i.e.*, antisense, ribozymes, and triplex-forming molecules), there are examples of natural products that interfere with deoxyribonucleic acid ("DNA") or RNA processes such as transcription or translation. For example, certain carbohydrate-based host cell factors, calicheamicin oligosaccharides, interfere with the sequence-specific binding of transcription factors to DNA and inhibit transcription *in vivo* (Ho *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:9203-9207; Liu *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93:940-944). Certain classes of known antibiotics have been characterized and were found to interact with RNA. For example, the antibiotic thiostreptone binds tightly to a 60-mer from ribosomal RNA (Cundliffe *et al.*, 1990, in The Ribosome: Structure, Function & Evolution (Schlessinger *et al.*, eds.) American Society for Microbiology, Washington, D.C. pp. 479-490). Bacterial resistance to various antibiotics often involves methylation at specific rRNA sites (Cundliffe, 1989, Ann. Rev. Microbiol. 43:207-233). Aminoglycosidic aminocyclitol (aminoglycoside) antibiotics and peptide antibiotics are known to inhibit group I intron splicing by binding to specific regions of the RNA (von Ahsen *et al.*, 1991, Nature (London) 353:368-370). Some of these same aminoglycosides have also been found to inhibit hammerhead ribozyme function (Stage *et al.*, 1995, RNA 1:95-101). In addition, certain aminoglycosides and other protein synthesis inhibitors have been found to interact with specific bases in 16S rRNA (Woodcock *et al.*, 1991, EMBO J. 10:3099-3103). An oligonucleotide analog of the 16S rRNA has also been shown to interact with certain aminoglycosides (Purohit *et al.*, 1994, Nature 370:659-662). A molecular basis for hypersensitivity to aminoglycosides has been found to be located in a single base change in mitochondrial rRNA (Hutchin *et al.*, 1993, Nucleic Acids Res. 21:4174-4179). Aminoglycosides have also been shown to inhibit the interaction between specific structural RNA motifs and the corresponding RNA binding protein. Zapp *et al.* (Cell, 1993, 74:969-978) has demonstrated that the aminoglycosides neomycin B, lividomycin A, and tobramycin can block the binding of Rev, a viral regulatory protein required for viral gene expression, to its viral recognition element in the IIB (or RRE) region of HIV RNA. This blockage appears to be the result of competitive binding of the antibiotics directly to the RRE RNA structural motif.

Single stranded sections of RNA can fold into complex tertiary structures consisting of local motifs such as loops, bulges, pseudoknots, guanosine quartets and turns (Chastain & Tinoco, 1991, Progress in Nucleic Acid Res. & Mol. Biol. 41:131-177; Chow & Bogdan, 1997, Chemical Reviews 97:1489-1514; Rando & Hogan, 1998, Biologic activity of guanosine quartet forming oligonucleotides in "Applied Antisense Oligonucleotide Technology" Stein. & Krieg (eds) John Wiley and Sons, New York, pages

335-352). Such structures can be critical to the activity of the nucleic acid and affect functions such as regulation of mRNA transcription, stability, or translation (Weeks & Crothers, 1993, Science 261:1574-1577). The dependence of these functions on the native three-dimensional structural motifs of single-stranded stretches of nucleic acids makes it difficult to identify or design synthetic agents that bind to these motifs using general, simple-to-use sequence-specific recognition rules for the formation of double- and triple-helical nucleic acids used in the design of antisense and ribozyme type molecules. Approaches to screening generally involve competitive assays designed to identify compounds that disrupt the interaction between a target RNA and a physiological, host cell factor(s) that had been previously identified to specifically interact with that particular target RNA. In general, such assays require the identification and characterization of the host cell factor(s) deemed to be required for the function of the target RNA. Both the target RNA and its preselected host cell binding partner are used in a competitive format to identify compounds that disrupt or interfere with the two components in the assay.

Citation or identification of any reference in Section 2 of this application is not an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention relates to methods for identifying compounds that bind to preselected target elements of nucleic acids including, but not limited to, specific RNA sequences, RNA structural motifs, and/or RNA structural elements. The specific target RNA sequences, RNA structural motifs, and/or RNA structural elements are used as targets for screening small molecules and identifying those that directly bind these specific sequences, motifs, and/or structural elements. For example, methods are described in which a preselected target RNA having a detectable label is used to screen a library of test compounds, preferably under physiologic conditions. Any complexes formed between the target RNA and a member of the library are identified using physical methods that detect the altered physical property of the target RNA bound to a test compound. In particular, the present invention relates to methods for using a target RNA having a detectable label to screen a library of test compounds free in solution, in labeled tubes or microtiter plate, or in a microarray. Compounds in the library that bind to the labeled target RNA will form a detectably labeled complex. The detectably labeled complex can then be identified and removed from the uncomplexed, unlabeled test compounds in the library, and from uncomplexed, labeled target RNA, by a variety of methods, including but not limited to, methods that differentiate changes in the electrophoretic, chromatographic, or thermostable

properties of the complexed target RNA. Such methods include, but are not limited to, electrophoresis, fluorescence spectroscopy, surface plasmon resonance, mass spectrometry, scintillation, proximity assay, structure-activity relationships ("SAR") by NMR spectroscopy, size exclusion chromatography, affinity chromatography, and nanoparticle aggregation. The structure of the test compound attached to the labeled RNA is then determined. The methods used will depend, in part, on the nature of the library screened. For example, assays or microarrays of test compounds, each having an address or identifier, may be deconvoluted, *e.g.*, by cross-referencing the positive sample to original compound list that was applied to the individual test assays. Another method for identifying test compounds includes *de novo* structure determination of the test compounds using mass spectrometry or nuclear magnetic resonance ("NMR"). The test compounds identified are useful for any purpose to which a binding reaction may be put, for example in assay methods, diagnostic procedures, cell sorting, as inhibitors of target molecule function, as probes, as sequestering agents and the like. In addition, small organic molecules which interact specifically with target RNA molecules may be useful as lead compounds for the development of therapeutic agents.

The methods described herein for the identification of compounds that directly bind to a particular preselected target RNA are well suited for high-throughput screening. The direct binding method of the invention offers advantages over drug screening systems for competitors that inhibit the formation of naturally-occurring RNA binding protein:target RNA complexes; *i.e.*, competitive assays. The direct binding method of the invention is rapid and can be set up to be readily performed, *e.g.*, by a technician, making it amenable to high throughput screening. The method of the invention also eliminates the bias inherent in the competitive drug screening systems, which require the use of a preselected host cell factor that may not have physiological relevance to the activity of the target RNA. Instead, the methods of the invention are used to identify any compound that can directly bind to specific target RNA sequences, RNA structural motifs, and/or RNA structural elements, preferably under physiologic conditions. As a result, the compounds so identified can inhibit the interaction of the target RNA with any one or more of the native host cell factors (whether known or unknown) required for activity of the RNA *in vivo*.

The present invention may be understood more fully by reference to the detailed description and examples, which are intended to illustrate non-limiting embodiments of the invention.

3.1. Definitions

As used herein, a "target nucleic acid" refers to RNA, DNA, or a chemically modified variant thereof. In a preferred embodiment, the target nucleic acid is RNA. A target nucleic acid also refers to tertiary structures of the nucleic acids, such as, but not limited to loops, bulges, pseudoknots, guanosine quartets and turns. A target nucleic acid also refers to RNA elements such as, but not limited to, the HIV TAR element, internal ribosome entry site, "slippery site", instability elements, and adenylate uridylate-rich elements, which are described in Section 5.1. Non-limiting examples of target nucleic acids are presented in Section 5.1 and Section 6.

As used herein, a "library" refers to a plurality of test compounds with which a target nucleic acid molecule is contacted. A library can be a combinatorial library, *e.g.*, a collection of test compounds synthesized using combinatorial chemistry techniques, or a collection of unique chemicals of low molecular weight (less than 1000 daltons) that each occupy a unique three-dimensional space.

As used herein, a "label" or "detectable label" is a composition that is detectable, either directly or indirectly, by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include radioactive isotopes (*e.g.*, ^{32}P, ^{35}S, and ^{3}H), dyes, fluorescent dyes, electron-dense reagents, enzymes and their substrates (*e.g.*, as commonly used in enzyme-linked immunoassays, *e.g.*, alkaline phosphatase and horse radish peroxidase), biotin-streptavidin, digoxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available. Moreover, a label or detectable moiety can include a "affinity tag" that, when coupled with the target nucleic acid and incubated with a test compound or compound library, allows for the affinity capture of the target nucleic acid along with molecules bound to the target nucleic acid. One skilled in the art will appreciate that a affinity tag bound to the target nucleic acids has, by definition, a complimentary ligand coupled to a solid support that allows for its capture. For example, useful affinity tags and complimentary partners include, but are not limited to, biotin-streptavidin, complimentary nucleic acid fragments (e.g., oligo dT-oligo dA, oligo T-oligo A, oligo dG-oligo dC, oligo G-oligo C), aptamers, or haptens and proteins for which antisera or monoclonal antibodies are available. The label or detectable moiety is typically bound, either covalently, through a linker or chemical bound, or through ionic, van der Waals or hydrogen bonds to the molecule to be detected.

As used herein, a "dye" refers to a molecule that, when exposed to radiation, emits radiation at a level that is detectable visually or via conventional spectroscopic means.

As used herein, a "visible dye" refers to a molecule having a chromophore that absorbs radiation in the visible region of the spectrum (*i.e.*, having a wavelength of between about 400 nm and about 700 nm) such that the transmitted radiation is in the visible region and can be detected either visually or by conventional spectroscopic means. As used herein, an "ultraviolet dye" refers to a molecule having a chromophore that absorbs radiation in the ultraviolet region of the spectrum (*i.e.*, having a wavelength of between about 30 nm and about 400 nm). As used herein, an "infrared dye" refers to a molecule having a chromophore that absorbs radiation in the infrared region of the spectrum (*i.e.*, having a wavelength between about 700 nm and about 3,000 nm). A "chromophore" is the network of atoms of the dye that, when exposed to radiation, emits radiation at a level that is detectable visually or via conventional spectroscopic means. One of skill in the art will readily appreciate that although a dye absorbs radiation in one region of the spectrum, it may emit radiation in another region of the spectrum. For example, an ultraviolet dye may emit radiation in the visible region of the spectrum. One of skill in the art will also readily appreciate that a dye can transmit radiation or can emit radiation via fluorescence or phosphorescence.

The phrase "pharmaceutically acceptable salt(s)," as used herein includes but is not limited to salts of acidic or basic groups that may be present in test compounds identified using the methods of the present invention. Test compounds that are basic in nature are capable of forming a wide variety of salts with various inorganic and organic acids. The acids that can be used to prepare pharmaceutically acceptable acid addition salts of such basic compounds are those that form non-toxic acid addition salts, *i.e.*, salts containing pharmacologically acceptable anions, including but not limited to sulfuric, citric, maleic, acetic, oxalic, hydrochloride, hydrobromide, hydroiodide, nitrate, sulfate, bisulfate, phosphate, acid phosphate, isonicotinate, acetate, lactate, salicylate, citrate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucaronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate and pamoate (*i.e.*, 1,1'-methylene-bis-(2-hydroxy-3-naphthoate)) salts. Test compounds that include an amino moiety may form pharmaceutically or cosmetically acceptable salts with various amino acids, in addition to the acids mentioned above. Test compounds that are acidic in nature are capable of forming base salts with various pharmacologically or cosmetically acceptable cations. Examples of such salts include alkali metal or alkaline earth metal salts and, particularly, calcium, magnesium, sodium lithium, zinc, potassium, and iron salts.

By "substantially one type of test compound," as used herein, is meant that the assay can be performed in such a fashion that at some point, only one compound need be used in each reaction so that, if the result is indicative of a binding event occurring between the target RNA molecule and the test compound, the test compound can be easily identified.

4. DESCRIPTION OF DRAWINGS

FIG. 1. Gel retardation analysis to detect peptide-RNA interactions. In 20 μl reactions containing increasing concentrations of Tat_{47-58} peptide (0.1 μM, 0.2 μM, 0.4 μM, 0.8 μM, 1.6 μM) 50 pmole TAR RNA oligonucleotide was added in TK buffer. The reaction mixture was then heated at 90 °C for 2 min and allowed to cool slowly to 24 °C. 10 ml of 30% glycerol was added to each sample and applied to a 12% non-denaturing polyacrylamide gel. The gel was electrophoresed using 1200 volt-hours at 4 °C in TBE Buffer. Following electrophoresis, the gel was dried and the radioactivity was quantitated with a phosphorimager. The concentration of peptide added is indicated above each lane.

FIG. 2. Gentamicin interacts with an oligonucleotide corresponding to the 16S rRNA. 20 μl reactions containing increasing concentrations of gentamicin (1 ng/ml, 10 ng/ml, 100 ng/ml, 1 μg/ml, 10 μg/ml, 50 μg/ml, 500 μg/ml) were added to 50 pmole RNA oligonucleotide in TKM buffer, heated at 90 °C for 2 min and allowed to cool slowly to 24 °C. Then 10 μl of 30% glycerol was added to each sample and the samples were applied to a 13.5% non-denaturing polyacrylamide gel. The gel was electrophoresed using 1200 volt-hours at 4 °C in TBE Buffer. Following electrophoresis, the gel was dried and the radioactivity was quantitated using a phosphorimager. The concentration of gentamicin added is indicated above each lane.

FIG. 3. The presence of 10 pg/ml gentamicin produces a gel mobility shift in the presence of the 16S rRNA oligonucleotide. 20 μl reactions containing increasing concentrations of gentamicin (100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml, and 10 pg/ml) were added to 50 pmole RNA oligonucleotide in TKM buffer were treated as described for Figure 2.

FIG. 4. Gentamicin binding to the 16S rRNA oligonucleotide is weak in the absence of $MgCl_2$. Reaction mixtures containing gentamicin (1 mg/ml, 100 μg/ml,

10 μg/ml,1 μg/ml, 0.1 μg/ml, and 10 ng/ml) were treated as described in Figure 2 except that the TKM buffer does not contain $MgCl_2$.

FIG. 5. Gel retardation analysis to detect peptide-RNA interactions. In reactions containing increasing concentrations of Tat_{47-58} peptide (0.1 μM, 0.2 μM, 0.4 μM, 0.8 μM, 1.6 μM) 50 pmole TAR RNA oligonucleotide was added in TK buffer. The reaction mixture was then heated at 90°C for 2 min and allowed to cool slowly to 24°C. The reactions were loaded onto a SCE9610 automated capillary electrophoresis apparatus (SpectruMedix; State College, Pennsylvania). The peaks correspond to the amount of free TAR RNA ("TAR") or the Tat-TAR complex ("Tat-TAR"). The concentration of peptide added is indicated below each lane.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for identifying compounds that bind to preselected target elements of nucleic acids, in particular, RNAs, including but not limited to preselected target RNA sequencing structural motifs, or structural elements. Methods are described in which a preselected target RNA having a detectable label is used to screen a library of test compounds. Any complexes formed between the target RNA and a member of the library are identified using physical methods that detect the altered physical property of the target RNA bound to a test compound. Changes in the physical property of the RNA-test compound complex relative to the target RNA or test compound can be measured by methods such as, but not limited to, methods that detect a change in mobility due to a change in mass, change in charge, or a change in thermostability. Such methods include, but are not limited to, electrophoresis, fluorescence spectroscopy, surface plasmon resonance, mass spectrometry, scintillation, proximity assay, structure-activity relationships ("SAR") by NMR spectroscopy, size exclusion chromatography, affinity chromatography, and nanoparticle aggregation. In particular, the present invention relates to methods for using a target RNA having a detectable label to screen a library of test compounds free in solution, in labeled tubes or microtiter plate, or in a microarray. Compounds in the library that bind to the labeled target RNA will form a detectably labeled complex. The detectably labeled complex can then be identified and removed from the unlabeled, uncomplexed test compounds in the library by a variety of methods capable of differentiating changes in the physical properties of the complexed target RNA. The structure of the test compound attached to the labeled RNA is also determined. The methods used will depend, in part, on the nature of the library screened. For example, assays or microarrays of test compounds,

each having an address or identifier, may be deconvoluted, *e.g.*, by cross-referencing the positive sample to an original compound list that was applied to the individual test assays. Another method for identifying test compounds includes *de novo* structure determination of the test compounds using mass spectrometry or nuclear magnetic resonance ("NMR").

Thus, the methods of the present invention provide a simple, sensitive assay for high-throughput screening of libraries of test compounds, in which the test compounds of the library that specifically bind a preselected target nucleic acid are easily distinguished from non-binding members of the library. The structures of the binding molecules are deciphered from the input library by methods depending on the type of library that is used. The test compounds so identified are useful for any purpose to which a binding reaction may be put, for example in assay methods, diagnostic procedures, cell sorting, as inhibitors of target molecule function, as probes, as sequestering agents and lead compounds for development of therapeutics, and the like. Small organic compounds that are identified to interact specifically with the target RNA molecules are particularly attractive candidates as lead compounds for the development of therapeutic agents.

The assay of the invention reduces bias introduced by competitive binding assays which require the identification and use of a host cell factor (presumably essential for modulating RNA function) as a binding partner for the target RNA. The assays of the present invention are designed to detect any compound or agent that binds to the target RNA, preferably under physiologic conditions. Such agents can then be tested for biological activity, without establishing or guessing which host cell factor or factors is required for modulating the function and/or activity of the target RNA.

Section 5.1 describes examples of protein-RNA interactions that are important in a variety of cellular functions and several target RNA elements that can be used to identify test compounds. Compounds that inhibit these interactions by binding to the RNA and successfully competing with the natural protein or host cell factor that endogenously binds to the RNA may be important, *e.g.*, in treating or preventing a disease or abnormal condition, such as an infection or unchecked growth. Section 5.2 describes detectable labels for target nucleic acids that are useful in the methods of the invention. Section 5.3 describes libraries of test compounds. Section 5.4 provides conditions for binding a labeled target RNA to a test compound of a library and detecting RNA binding to a test compound using the methods of the invention. Section 5.5 provides methods for separating complexes of target RNAs bound to a test compound from an unbound RNA. Section 5.6 describes methods for identifying test compounds that are bound to the target RNA. Section 5.7 describes a secondary, biological screen of test compounds identified by

the methods of the invention to test the effect of the test compounds *in vivo*. Section 5.8 describes the use of test compounds identified by the methods of the invention for treating or preventing a disease or abnormal condition in mammals.

5.1. Biologically Important RNA-Host Cell Factor Interactions

Nucleic acids, and in particular RNAs, are capable of folding into complex tertiary structures that include bulges, loops, triple helices and pseudoknots, which can provide binding sites for host cell factors, such as proteins and other RNAs. RNA-protein and RNA-RNA interactions are important in a variety cellular functions, including transcription, RNA splicing, RNA stability and translation. Furthermore, the binding of such host cell factors to RNAs may alter the stability and translational efficiency of such RNAs, and according affect subsequent translation. For example, some diseases are associated with protein overproduction or decreased protein function. In this case, the identification of compounds to modulate RNA stability and translational efficiency will be useful to treat and prevent such diseases.

The methods of the present invention are useful for identifying test compounds that bind to target RNA elements in a high throughput screening assay of libraries of test compounds in solution. In particular, the methods of the present invention are useful for identifying a test compound that binds to a target RNA elements and inhibits the interaction of that RNA with one or more host cell factors *in vivo*. The molecules identified using the methods of the invention are useful for inhibiting the formation of a specific bound RNA:host cell factor complexes *in vivo*.

In some embodiments, test compounds identified by the methods of the invention are useful for increasing or decreasing the translation of messenger RNAs ("mRNAs"), *e.g.*, protein production, by binding to one or more regulatory elements in the 5' untranslated region, the 3' untranslated region, or the coding region of the mRNA. Compounds that bind to mRNA can, *inter alia*, increase or decrease the rate of mRNA processing, alter its transport through the cell, prevent or enhance binding of the mRNA to ribosomes, suppressor proteins or enhancer proteins, or alter mRNA stability. Accordingly, compounds that increase or decrease mRNA translation can be used to treat or prevent disease. For example, diseases associated with protein overproduction, such as amyloidosis, or with the production of mutant proteins, such as *Ras*, can be treated or prevented by decreasing translation of the mRNA that codes for the overproduced protein, thus inhibiting production of the protein. Conversely, the symptoms of diseases associated with decreased protein function, such as hemophelia, may be treated by increasing

translation of mRNA coding for the protein whose function is decreased, *e.g.*, factor IX in some forms of hemophilia.

The methods of the invention can be used to identify compounds that bind to mRNAs coding for a variety of proteins with which the progression of diseases in mammals is associated. These mRNAs include, but are not limited to, those coding for amyloid protein and amyloid precursor protein; anti-angiogenic proteins such as angiostatin, endostatin, METH-1 and METH-2; apoptosis inhibitor proteins such as survivin, clotting factors such as Factor IX, Factor VIII, and others in the clotting cascade; collagens; cyclins and cyclin inhibitors, such as cyclin dependent kinases, cyclin D1, cyclin E, WAF1, cdk4 inhibitor, and MTS1; cystic fibrosis transmembrane conductance regulator gene (CFTR); cytokines such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17 and other interleukins; hematopoetic growth factors such as erythropoietin (Epo); colony stimulating factors such as G-CSF, GM-CSF, M-CSF, SCF and thrombopoietin; growth factors such as BNDF, BMP, GGRP, EGF, FGF, GDNF, GGF, HGF, IGF-1, IGF-2, KGF, myotrophin, NGF, OSM, PDGF, somatotrophin, TGF-ß, TGF-α and VEGF; antiviral cytokines such as interferons, antiviral proteins induced by interferons, TNF-α, and TNF-ß; enzymes such as cathepsin K, cytochrome P-450 and other cytochromes, farnesyl transferase, glutathione-S transferases, heparanase, HMG CoA synthetase, N-acetyltransferase, phenylalanine hydroxylase, phosphodiesterase, ras carboxyl-terminal protease, telomerase and TNF converting enzyme; glycoproteins such as cadherins, *e.g.*, N-cadherin and E-cadherin; cell adhesion molecules; selectins; transmembrane glycoproteins such as CD40; heat shock proteins; hormones such as 5-α reductase, atrial natriuretic factor, calcitonin, corticotrophin releasing factor, diuretic hormones, glucagon, gonadotropin, gonadotropin releasing hormone, growth hormone, growth hormone releasing factor, somatotropin, insulin, leptin, luteinizing hormone, luteinizing hormone releasing hormone, parathyroid hormone, thyroid hormone, and thyroid stimulating hormone; proteins involved in immune responses, including antibodies, CTLA4, hemagglutinin, MHC proteins, VLA-4, and kallikrein-kininogen-kinin system; ligands such as CD4; oncogene products such as *sis*, *hst*, protein tyrosine kinase receptors, *ras*, *abl*, *mos*, *myc*, *fos*, *jun*, *H-ras*, *ki-ras*, *c-fms*, *bcl-2*, *L-myc*, *c-myc*, *gip*, *gsp*, and *HER-2*; receptors such as bombesin receptor, estrogen receptor, GABA receptors, growth factor receptors including EGFR, PDGFR, FGFR, and NGFR, GTP-binding regulatory proteins, interleukin receptors, ion channel receptors, leukotriene receptor antagonists, lipoprotein receptors, opioid pain receptors, substance P receptors, retinoic acid and retinoid receptors, steroid receptors, T-cell receptors, thyroid hormone receptors, TNF receptors; tissue

plasminogen activator; transmembrane receptors; transmembrane transporting systems, such as calcium pump, proton pump, Na/Ca exchanger, MRP1, MRP2, P170, LRP, and cMOAT; transferrin; and tumor suppressor gene products such as *APC, brca1, brca2, DCC, MCC, MTS1, NF1, NF2, nm23, p53* and *Rb.* In addition to the eukaryotic genes listed above, the invention, as described, can be used to define molecules that interrupt viral, bacterial or fungal transcription or translation efficiencies and therefore form the basis for a novel anti-infectious disease therapeutic. Other target genes include, but are not limited to, those disclosed in Section 5.1 and Section 6.

The methods of the invention can be used to identify mRNA-binding test compounds for increasing or decreasing the production of a protein, thus treating or preventing a disease associated with decreasing or increasing the production of said protein, respectively. The methods of the invention may be useful for identifying test compounds for treating or preventing a disease in mammals, including cats, dogs, swine, horses, goats, sheep, cattle, primates and humans. Such diseases include, but are not limited to, amyloidosis, hemophilia, Alzheimer's disease, atherosclerosis, cancer, giantism, dwarfism, hypothyroidism, hyperthyroidism, inflammation, cystic fibrosis, autoimmune disorders, diabetes, aging, obesity, neurodegenerative disorders, and Parkinson's disease. Other diseases include, but are not limited to, those described in Section 5.1 and diseases caused by aberrant expression of the genes disclosed in Example 6. In addition to the eukaryotic genes listed above, the invention, as described, can be used to define molecules that interrupt viral, bacterial or fungal transcription or translation efficiencies and therefore form the basis for a novel anti-infectious disease therapeutic.

In other embodiments, test compounds identified by the methods of the invention are useful for preventing the interaction of an RNA, such as a transfer RNA ("tRNA"), an enzymatic RNA or a ribosomal RNA ("rRNA"), with a protein or with another RNA, thus preventing, *e.g.*, assembly of an *in vivo* protein-RNA or RNA-RNA complex that is essential for the viability of a cell. The term "enzymatic RNA," as used herein, refers to RNA molecules that are either self-splicing, or that form an enzyme by virtue of their association with one or more proteins, *e.g.*, as in RNase P, telomerase or small nuclear ribonuclear protein particles. For example, inhibition of an interaction between rRNA and one or more ribosomal proteins may inhibit the assembly of ribosomes, rendering a cell incapable of synthesizing proteins. In addition, inhibition of the interaction of precursor rRNA with ribonucleases or ribonucleoprotein complexes (such as RNase P) that process the precursor rRNA prevent maturation of the rRNA and its assembly into ribosomes. Similarly, a tRNA:tRNA synthetase complex may be inhibited by test

compounds identified by the methods of the invention such that tRNA molecules do not become charged with amino acids. Such interactions include, but are not limited to, rRNA interactions with ribosomal proteins, tRNA interactions with tRNA synthetase, RNase P protein interactions with RNase P RNA, and telomerase protein interactions with telomerase RNA.

In other embodiments, test compounds identified by the methods of the invention are useful for treating or preventing a viral, bacterial, protozoan or fungal infection. For example, transcriptional up-regulation of the genes of human immunodeficiency virus type 1 ("HIV-1") requires binding of the HIV Tat protein to the HIV trans-activation response region RNA ("TAR RNA"). HIV TAR RNA is a 59-base stem-loop structure located at the 5'-end of all nascent HIV-1 transcripts (Jones & Peterlin, 1994, Annu. Rev. Biochem. 63:717-43). Tat protein is known to interact with uracil 23 in the bulge region of the stem of TAR RNA. Thus, TAR RNA is a potential binding target for test compounds, such as small peptides and peptide analogs that bind to the bulge region of TAR RNA and inhibit formation of a Tat-TAR RNA complex involved in HIV-1 upregulation (see Hwang *et al.*,1999 Proc. Natl. Acad. Sci. USA 96:12997-13002). Accordingly, test compounds that bind to TAR RNA are useful as anti-HIV therapeutics (Hamy *et al.*, 1997, Proc. Natl. Acad. Sci. USA 94:3548-3553; Hamy *et al.*, 1998, Biochemistry 37:5086-5095; Mei *et al.*, 1998, Biochemistry 37:14204-14212), and therefore, are useful for treating or preventing AIDS.

The methods of the invention can be used to identify test compounds to treat or prevent viral, bacterial, protozoan or fungal infections in a patient. In some embodiments, the methods of the invention are useful for identifying compounds that decrease translation of microbial genes by interacting with mRNA, as described above, or for identifying compounds that inhibit the interactions of microbial RNAs with proteins or other ligands that are essential for viability of the virus or microbe. Examples of microbial target RNAs useful in the present invention for identifying antiviral, antibacterial, anti-protozoan and anti-fungal compounds include, but are not limited to, general antiviral and anti-inflammatory targets such as mRNAs of INFα, INFγ, RNAse L, RNAse L inhibitor protein, PKR, tumor necrosis factor, interleukins 1-15, and IMP dehydrogenase; internal ribosome entry sites; HIV-1 CT rich domain and RNase H mRNA; HCV internal ribosome entry site (required to direct translation of HCV mRNA), and the 3'-untranslated tail of HCV genomes; rotavirus NSP3 binding site, which binds the protein NSP3 that is required for rotavirus mRNA translation; HBV epsilon domain; Dengue virus 5' and 3' untranslated regions, including IRES; INFα, INFβ and INFγ; plasmodium falciparum mRNAs; the 16S

ribosomal subunit ribosomal RNA and the RNA component of RNase P of bacteria; and the RNA component of telomerase in fungi and cancer cells. Other target viral and bacterial mRNAs include, but are not limited to, those disclosed in Section 6.

One of skill in the art will appreciate that, although such target RNAs are functionally conserved in various species (*e.g.*, from yeast to humans), they exhibit nucleotide sequence and structural diversity. Therefore, inhibition of, for example, yeast telomerase by an anti-fungal compound identified by the methods of the invention might not interfere with human telomerase and normal human cell proliferation.

Thus, the methods of the invention can be used to identify test compounds that interfere with one or more target RNA interactions with host cell factors that are important for cell growth or viability, or essential in the life cycle of a virus, a bacterium, a protozoa or a fungus. Such test compounds and/or congeners that demonstrate desirable biologic and pharmacologic activity can be administered to a patient in need thereof in order to treat or prevent a disease caused by viral, bacterial, protozoan, or fungal infections. Such diseases include, but are not limited to, HIV infection, AIDS, human T-cell leukemia, SIV infection, FIV infection, feline leukemia, hepatitis A, hepatitis B, hepatitis C, Dengue fever, malaria, rotavirus infection, severe acute gastroenteritis, diarrhea, encephalitis, hemorrhagic fever, syphilis, legionella, whooping cough, gonorrhea, sepsis, influenza, pneumonia, tinea infection, candida infection, and meningitis.

Non-limiting examples of RNA elements involved in the regulation of gene expression, *i.e.*, mRNA stability, translational efficiency via translational initiation and ribosome assembly, *etc.*, include the HIV TAR element, internal ribosome entry site, "slippery site", instability elements, and adenylate uridylate-rich elements, as discussed below.

5.1.1. HIV TAR Element

Transcriptional up-regulation of the genes of human immunodeficieney virus type 1 ("HIV-1") requires binding of the HIV Tat protein to the HIV trans-activation response region RNA ("TAR RNA"), a 59-base stem-loop structure located at the 5' end of all nascent HIV-1 transcripts (Jones & Peterlin, 1994, Annu. Rev. Biochem. 63:717-43). Tat protein is known to interact with uracil 23 in the bulge region of the stem of TAR RNA. Thus, TAR RNA is a useful binding target for test compounds, such as small peptides and peptide analogs that bind to the bulge region of TAR RNA and inhibit formation of a Tat-TAR RNA complex involved in HIV-1 up-regulation (see Hwang *et al.*,1999 Proc. Natl. Acad. Sci. USA 96:12997-13002). Accordingly, test compounds that bind to TAR RNA

can be useful as anti-HIV therapeutics (Hamy *et al.*, 1997, Proc. Natl. Acad. Sci. USA 94:3548-3553; Hamy *et al.*, 1998, Biochemistry 37:5086-5095; Mei *et al.*, 1998, Biochemistry 37:14204-14212), and therefore, are useful for treating or preventing AIDS.

5.1.2. Internal Ribosome Entry Site ("IRES")

Internal ribosome entry sites ("IRES") are found in the 5' untranslated regions ("5' UTR") of several mRNAs, and are thought to be involved in the regulation of translational efficiency. When the IRES element is present on an mRNA downstream of a translational stop codon, it directs ribosomal re-entry (Ghattas *et al.*, 1991, Mol. Cell. Biol. 11:5848-5959), which permits initiation of translation at the start of a second open reading frame.

As reviewed by Jang *et al.*, a large segment of the 5' nontranslated region, approximately 400 nucleotides in length, promotes internal entry of ribosomes independent of the non-capped 5' end of picornavirus mRNAs (mammalian plus-strand RNA viruses whose genomes serve as mRNA). This 400 nucleotide segment (IRES), maps approximately 200 nt down-stream from the 5' end and is highly structured. IRES elements of different picornaviruses, although functionally similar *in vitro* and *in vivo*, are not identical in sequence or structure. However, IRES elements of the genera entero- and rhinoviruses, on the one hand, and cardio- and aphthoviruses, on the other hand, reveal similarities corresponding to phylogenetic kinship. All IRES elements contain a conserved Yn-Xm-AUG unit (Y, pyrimidine; X, nucleotide) which appears essential for IRES function. The IRES elements of cardio-, entero- and aphthoviruses bind a cellular protein, p57. In the case of cardioviruses, the interaction between a specific stem-loop of the IREs is essential for translation in vitro. The IRES elements of entero- and cardioviruses also bind the cellular protein, p52, but the significance of this interaction remains to be shown. The function of p57 or p52 in cellular metabolism is unknown. Since picornaviral IRES elements function *in vivo* in the absence of any viral gene products, is speculated that IRES-like elements may also occur in specific cellular mRNAs releasing them from cap-dependent translation (Jang *et al.*, 1990, Enzyme 44(1-4):292-309).

5.1.3. "Slippery Site"

Programmed, or directed, ribosomal frameshifting, when ribosomes shift from one translation reading frame to another and synthesize two viral proteins from a single viral mRNA, is directed by a unique site in viral mRNAs called the "slippery site." The slippery site directs ribosomal frameshifting in the -1 or +1 direction that causes the

ribosome to slip by one base in the 5' direction thereby placing the ribosome in the new reading frame to produce a new protein.

Programmed, or directed, ribosomal frameshifting is of particular value to viruses that package their plus strands, as it eliminates the need to splice their mRNAs and reduces the risk of packaging defective genomes and regulates the ratio of viral proteins synthesized. Examples of programmed translational frameshifting (both +1 and -1 shifts) have been identified in ScV systems (Lopinski *et al.*, 2000, Mol. Cell. Biol. 20(4):1095-103, retroviruses (Falk *et al.*, 1993, J. Virol. 67:273-6277; Jacks & Varmus, 1985, Science 230:1237-1242; Morikawa & Bishop, 1992, Virology 186:389-397; Nam *et al.*, 1993, J. Virol. 67:196-203); coronaviruses (Brierley *et al.*, 1987, EMBO J. 6:3779-3785; Herold & Siddell, 1993, Nucleic Acids Res. 21:5838-5842); giardiaviruses, which are also members of the Totiviridae (Wang *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:8595-8599); two bacterial genes (Blinkowa & Walker, 1990, Nucleic Acids Res., 18:1725-1729; Craigen & Caskey, 1986, Nature 322:273); bacteriophage genes (Condron *et al.*, 1991, Nucleic Acids Res. 19:5607-5612); astroviruses (Marczinke *et al.*, 1994, J. Virol. 68:5588-5595); the yeast EST3 gene (Lundblad & Morris, 1997, Curr. Biol. 7:969-976); and the rat, mouse, Xenopus, and Drosophila ornithine decarboxylase antizymes (Matsufuji *et al.*, 1995, Cell 80:51-60); and a significant number of cellular genes (Herold & Siddell, 1993, Nucleic Acids Res. 21:5838-5842).

Drugs targeted to ribosomal frameshifting minimize the problem of virus drug resistance because this strategy targets a host cellular process rather than one introduced into the cell by the virus, which minimizes the ability of viruses to evolve drug-resistant mutants. Compounds that target the RNA elements involved in regulating programmed frameshifting should have several advantages, including (a) any selective pressure on the host cellular translational machinery to adapt to the drugs would have to occur at the host evolutionary time scale, which is on the order of millions of years, (b) ribosomal frameshifting is not used to express any host proteins, and (c) altering viral frameshifting efficiencies by modulating the activity of a host protein minimizing the likelihood that the virus will acquire resistance to such inhibition by mutations in its own genome.

5.1.4. <u>Instability Elements</u>

"Instability elements" may be defined as specific sequence elements that promote the recognition of unstable mRNAs by cellular turnover machinery. Instability

elements have been found within mRNA protein coding regions as well as untranslated regions.

Altering the control of stability of normal mRNAs may lead to disease. The alteration of mRNA stability has been implicated in diseases such as, but not limited to, cancer, immune disorders, heart disease, and fibrotic disorders.

There are several examples of mutations that delete instability elements which then result in stabilization of mRNAs that may be involved in the onset of cancer. In Burkitt's lymphoma, a portion of the c-*myc* proto-oncogene is translocated to an Ig locus, producing a form of the c-*myc* mRNA that is five times more stable (*see, e.g.*, Kapstein *et al.*, 1996, J. Biol. Chem. 271(31):18875-84). The highly oncogenic v-*fos* mRNA lacks the 3' UTR adenylate uridylate rich element ("ARE") that is found in the more labile and weakly oncogenic c-*fos* mRNA (*see, e.g.*, Schiavi *et al.*, 1992, Biochim Biophys Acta. 1114(2-3):95-106). Differences between the benign cervical lesions brought about by nonintegrated circular human papillomavirus type 16 and its integrated form, that lacks the 3' UTR ARE and correlates with cervical carcinomas, may be a consequence of stabilizing the E6/E7 transcripts encoding oncogenic proteins. Integration of the virus results in deletion of the ARE instability element, resulting in stabilizion of the transcripts and over-expression of the proteins (*see, e.g.*, Jeon & Lambert, 1995, Proc. Natl. Acad. Sci. USA 92(5):1654-8). Deletion of AREs from the 3' UTR of the IL-2 and IL-3 genes promotes increased stabilization of these mRNAs, high expression of these proteins, and leads to the formation of cancerous cells (*see, e.g.*, Stoecklin *et al.*, 2000, Mol. Cell. Biol. *20(11):3753-63*).

Mutations in trans-acting factors involved in mRNA turnover may also promote cancer. In monocytic tumors, the lymphokine GM-CSF mRNA is specifically stabilized as a consequence of an oncogenic lesion in a trans-acting factor that controls mRNA turnover rates. Furthermore, the normally unstable IL-3 transcript is inappropriately long-lived in mast tumor cells. Similarly, the labile GM-CSF mRNA is greatly stabilized in bladder carcinoma cells. *See, e.g.*, Bickel *et al.*, 1990, J. Immunol. 145(3):840-5.

The immune system is regulated by a large number of regulatory molecules that either activate or inhibit the immune response. It has now been clearly demonstrated that stability of the transcripts encoding these proteins are highly regulated. Altered regulation of these molecules leads to mis-regulation of this process and can result in drastic medical consequences. For example, recent results using transgenic mice have shown that mis-regulation of the stability of the important modulator TNFα mRNA leads to diseases

such as, but not limited to, rheumatoid arthritis and a Crohn's-like liver disease. *See, e.g.*, Clark, 2000, Arthritis Res. 2(3):172-4.

Smooth muscle in the heart is modulated by the β-adrenergic receptor, which in turn responds to the sympathetic neurotransmitter norepinephrine and the adrenal hormone epinephrine. Chronic heart failure is characterized by impairment of smooth muscle cells, which results, in part, from the more rapid decay of the β-adrenergic receptor mRNA. *See, e.g.*, Ellis & Frielle, 1999, Biochem. Biophys. Res. Commun. 258(3):552-8.

A large number of diseases result from over-expression of collagen. For example, cirrhosis results from damage to the liver as a consequence of cancer, viral infection, or alcohol abuse. Such damage causes mis-regulation of collagen expression, leading to the formation of large collagen deposits. Recent results indicate that the sizeable increase in collagen expression is largely attributable to stabilization of its mRNA. *See, e.g.*, Lindquist *et al.*, 2000, Am. J. Physiol. Gastrointest. Liver Physiol. 279(3):G471-6.

5.1.5. Adenylate Uridylate-rich Elements ("ARE")

Adenylate uridylate-rich elements ("ARE") are found in the 3' untranslated regions ("3' UTR") of several mRNAs, and involved in the turnover of mRNAs, such as but not limited to transcription factors, cytokines, and lymphokines. AREs may function both as stabilizing and destabilizing elements. ARE mRNAs are classified into five groups, depending on sequence (Bakheet *et al.*, 2001, Nucl. Acids Res. 29(1):246-254). An ongoing database at the web site http://rc.kfshrc.edu.sa/ared contains ARE-containing mRNAs and their cluster groups, which is incorporated by reference in its entirety. The ARE motifs are classified as follows:

Group I Cluster	(AUUUAUUUAUUUAUUUAUUUA)	SEQ ID NO: 1
Group II Cluster	(AUUUAUUUAUUUAUUUA) stretch	SEQ ID NO: 2
Group III Cluster	(WAUUUAUUUAUUUAW) stretch	SEQ ID NO: 3
Group IV Cluster	(WWAUUUAUUUAWW) stretch	SEQ ID NO: 4
Group V Cluster	(WWWWAUUUAWWWW) stretch	SEQ ID NO: 5

The ARE-mRNAs were clustered into five groups containing five, four, three and two pentameric repeats, while the last group contains only one pentamer within the 13-bp ARE pattern. Functional categories were assigned whenever possible according to NCBI-COG functional annotation (Tatusov *et al.*, 2001, Nucleic Acids Research, 29(1): 22-28), in addition to the categories: inflammation, immune response, development/differentiation, using an extensive literature search.

Group I contains many secreted proteins including GM-CSF, IL-1, IL-11, IL-12 and Gro-ß that affect the growth of hematopoietic and immune cells (Witsell & Schook, 1992, Proc. Natl Acad. Sci. USA, 89:4754–4758). Although TNFα is both a pro-inflammatory and anti-tumor protein, there is experimental evidence that it can act as a growth factor in certain leukemias and lymphomas (Liu *et al.*, 2000, J. Biol. Chem. 275:21086–21093).

Unlike Group I, Groups II–V contain functionally diverse gene families comprising immune response, cell cycle and proliferation, inflammation and coagulation, angiogenesis, metabolism, energy, DNA binding and transcription, nutrient transportation and ionic homeostasis, protein synthesis, cellular biogenesis, signal transduction, and apoptosis (Bakheet *et al.*, 2001, Nucl. Acids Res. 29(1):246-254).

Several groups have described ARE-binding proteins that influence the ARE-mRNA stability. Among the well-characterized proteins are the mammalian homologs of ELAV (embryonic lethal abnormal vision) proteins including AUF1, HuR and He1-N2 (Zhang *et al.*, 1993, Mol. Cell. Biol. 13:7652–7665; Levine *et al.*, 1993, Mol. Cell. Biol. 13:3494–3504: Ma *et al.*, 1996, J. Biol. Chem. 271:8144–8151). The zinc-finger protein tristetraprolin has been identified as another ARE-binding protein with destabilizing activity on TNFα, IL-3 and GM-CSF mRNAs (Stoecklin *et al.*, 2000, Mol. Cell. Biol. 20:3753–3763; Carballo *et al.*, 2000, Blood 95:1891–1899).

Since ARE-containing genes are clearly important in biological systems, including but not limited to a number of the early response genes that regulate cell proliferation and responses to exogenous agents, the identification of compounds that bind to one or more of the ARE clusters and potentially modulate the stability of the target RNA can potentially be of value as a therapeutic.

5.2. <u>Detectably Labeled Target RNAs</u>

Target nucleic acids, including but not limited to RNA and DNA, useful in the methods of the present invention have a label that is detectable via conventional spectroscopic means or radiographic means. Preferably, target nucleic acids are labeled with a covalently attached dye molecule. Useful dye-molecule labels include, but are not limited to, fluorescent dyes, phosphorescent dyes, ultraviolet dyes, infrared dyes, and visible dyes. Preferably, the dye is a visible dye.

Useful labels in the present invention can include, but are not limited to, spectroscopic labels such as fluorescent dyes (*e.g.*, fluorescein and derivatives such as fluorescein isothiocyanate (FITC) and Oregon Green™, rhodamine and derivatives (e.g.,

Texas red, tetramethylrhodimine isothiocynate (TRITC), bora-3a,4a-diaza-s-indacene (BODIPY®) and derivatives, *etc.*), digoxigenin, biotin, phycoerythrin, AMCA, CyDye™, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P, *etc.*), enzymes (e.g., horse radish peroxidase, alkaline phosphatase *etc.*), spectroscopic colorimetric labels such as colloidal gold or colored glass or plastic (e.g. polystyrene, polypropylene, latex, etc.) beads, or nanoparticles – nanoclusters of inorganic ions with defined dimension from 0.1 to 1000 nm. Useful affinity tags and complimentary partners include, but are not limited to, biotin-streptavidin, complimentary nucleic acid fragments (e.g., oligo dT-oligo dA, oligo T-oligo A, oligo dG-oligo dC, oligo G-oligo C), aptamer-streptavidin, or haptens and proteins for which antisera or monoclonal antibodies are available. The label may be coupled directly or indirectly to a component of the detection assay (*e.g.*, the detection reagent) according to methods well known in the art. A wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

In one embodiment, nucleic acids that are labeled at one or more specific locations are chemically synthesized using phosphoramidite or other solution or solid-phase methods. Detailed descriptions of the chemistry used to form polynucleotides by the phosphoramidite method are well known (*see, e.g.*, Caruthers *et al.*, U.S. Pat. Nos. 4,458,066 and 4,415,732; Caruthers *et al.*, 1982, Genetic Engineering 4:1-17; *Users Manual Model 392 and 394 Polynucleotide Synthesizers*, 1990, pages 6-1 through 6-22, Applied Biosystems, Part No. 901237; Ojwang, *et al.*, 1997, Biochemistry, 36:6033-6045). The phosphoramidite method of polynucleotide synthesis is the preferred method because of its efficient and rapid coupling and the stability of the starting materials. The synthesis is performed with the growing polynucleotide chain attached to a solid support, such that excess reagents, which are generally in the liquid phase, can be easily removed by washing, decanting, and/or filtration, thereby eliminating the need for purification steps between synthesis cycles.

The following briefly describes illustrative steps of a typical polynucleotide synthesis cycle using the phosphoramidite method. First, a solid support to which is attached a protected nucleoside monomer at its 3' terminus is treated with acid, *e.g.*, trichloroacetic acid, to remove the 5'-hydroxyl protecting group, freeing the hydroxyl group for a subsequent coupling reaction. After the coupling reaction is completed an activated intermediate is formed by contacting the support-bound nucleoside with a protected nucleoside phosphoramidite monomer and a weak acid, *e.g.*, tetrazole. The weak acid protonates the nitrogen atom of the phosphoramidite forming a reactive intermediate.

Nucleoside addition is generally complete within 30 seconds. Next, a capping step is performed, which terminates any polynucleotide chains that did not undergo nucleoside addition. Capping is preferably performed using acetic anhydride and 1-methylimidazole. The phosphite group of the internucleotide linkage is then converted to the more stable phosphotriester by oxidation using iodine as the preferred oxidizing agent and water as the oxygen donor. After oxidation, the hydroxyl protecting group of the newly added nucleoside is removed with a protic acid, *e.g.*, trichloroacetic acid or dichloroacetic acid, and the cycle is repeated one or more times until chain elongation is complete. After synthesis, the polynucleotide chain is cleaved from the support using a base, *e.g.*, ammonium hydroxide or t-butyl amine. The cleavage reaction also removes any phosphate protecting groups, *e.g.*, cyanoethyl. Finally, the protecting groups on the exocyclic amines of the bases and any protecting groups on the dyes are removed by treating the polynucleotide solution in base at an elevated temperature, *e.g.*, at about 55°C. Preferably the various protecting groups are removed using ammonium hydroxide or t-butyl amine.

Any of the nucleoside phosphoramidite monomers can be labeled using standard phosphoramidite chemistry methods (Hwang *et al.*, 1999, Proc. Natl. Acad. Sci. USA 96(23):12997-13002; Ojwang *et al.*, 1997, Biochemistry. 36:6033-6045 and references cited therein). Dye molecules useful for covalently coupling to phosphoramidites preferably comprise a primary hydroxyl group that is not part of the dye's chromophore. Illustrative dye molecules include, but are not limited to, disperse dye CAS 4439-31-0, disperse dye CAS 6054-58-6, disperse dye CAS 4392-69-2 (Sigma-Aldrich, St. Louis, MO), disperse red, and 1-pyrenebutanol (Molecular Probes, Eugene, OR). Other dyes useful for coupling to phosphoramidites will be apparent to those of skill in the art, such as fluoroscein, cy3, and cy5 fluorescent dyes, and may be purchased from, *e.g.*, Sigma-Aldrich, St. Louis, MO or Molecular Probes, Inc., Eugene, OR.

In another embodiment, dye-labeled target RNA molecules are synthesized enzymatically using *in vitro* transcription (Hwang *et al.*, 1999, Proc. Natl. Acad. Sci. USA 96(23):12997-13002 and references cited therein). In this embodiment, a template DNA is denatured by heating to about 90°C and an oligonucleotide primer is annealed to the template DNA, for example by slow-cooling the mixture of the denatured template and the primer from about 90°C to room temperature. A mixture of ribonucleoside-5'-triphosphates capable of supporting template-directed enzymatic extension of the primed template (*e.g.*, a mixture including GTP, ATP, CTP, and UTP), including one or more dye-labeled ribonucleotides (Sigma-Aldrich, St. Louis, MO), is added to the primed template. Next, a polymerase enzyme is added to the mixture under conditions where the polymerase enzyme

is active, which are well-known to those skilled in the art. A labeled polynucleotide is formed by the incorporation of the labeled ribonucleotides during polymerase-mediated strand synthesis.

In yet another embodiment of the invention, nucleic acid molecules are end-labeled after their synthesis. Methods for labeling the 5'-end of an oligonucleotide include but are by no means limited to: (i) periodate oxidation of a 5'-to-5'-coupled ribonucleotide, followed by reaction with an amine-reactive label (Heller & Morisson, 1985, in *Rapid Detection and Identification of Infectious Agents,* D.T. Kingsbury and S. Falkow, eds., pp. 245-256, Academic Press); (ii) condensation of ethylenediamine with 5'-phosphorylated polynucleotide, followed by reaction with an amine reactive label (Morrison, European Patent Application 232 967); (iii) introduction of an aliphatic amine substituent using an aminohexyl phosphite reagent in solid-phase DNA synthesis, followed by reaction with an amine reactive label (Cardullo *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:8790-8794); and (iv) introduction of a thiophosphate group on the 5'-end of the nucleic acid, using phosphatase treatment followed by end-labeling with ATP-?S and kinase, which reacts specifically and efficiently with maleimide-labeled fluorescent dyes (Czworkowski *et al.*, 1991, Biochem. 30:4821-4830).

A detectable label should not be incorporated into a target nucleic acid at the specific binding site at which test compounds are likely to bind, since the presence of a covalently attached label might interfere sterically or chemically with the binding of the test compounds at this site. Accordingly, if the region of the target nucleic acid that binds to a host cell factor is known, a detectable label is preferably incorporated into the nucleic acid molecule at one or more positions that are spatially or sequentially remote from the binding region.

After synthesis, the labeled target nucleic acid can be purified using standard techniques known to those skilled in the art (*see* Hwang *et al.*, 1999, Proc. Natl. Acad. Sci. USA 96(23):12997-13002 and references cited therein). Depending on the length of the target nucleic acid and the method of its synthesis, such purification techniques include, but are not limited to, reverse-phase high-performance liquid chromatography ("reverse-phase HPLC"), fast performance liquid chromatography ("FPLC"), and gel purification. After purification, the target RNA is refolded into its native conformation, preferably by heating to approximately 85-95°C and slowly cooling to room temperature in a buffer, *e.g.*, a buffer comprising about 50 mM Tris-HCl, pH 8 and 100 mM NaCl.

In another embodiment, the target nucleic acid can also be radiolabeled. A radiolabel, such as, but not limited to, an isotope of phosphorus, sulfur, or hydrogen, may be

incorporated into a nucleotide, which is added either after or during the synthesis of the target nucleic acid. Methods for the synthesis and purification of radiolabeled nucleic acids are well known to one of skill in the art. *See, e.g.,* Sambrook *et al.*, 1989, in *Molecular Cloning: A Laboratory Manual*, pp 10.2-10.70, Cold Spring Harbor Laboratory Press, and the references cited therein, which are hereby incorporated by reference in their entireties.

In another embodiment, the target nucleic acid can be attached to an inorganic nanoparticle. A nanoparticle is a cluster of ions with controlled size from 0.1 to 1000 nm comprised of metals, metal oxides, or semiconductors including, but not limited to Ag_2S, ZnS, CdS, CdTe, Au, or TiO_2. Nanoparticles have unique optical, electronic and catalytic properties relative to bulk materials which can be adjusted according to the size of the particle. Methods for the attachment of nucleic acids are well know to one of skill in the art (see, *e.g.*, Niemeyer, 2001, Angew. Chem. Int. Ed. 40: 4129-4158, International Patent Publication WO/0218643, and the references cited therein, the disclosures of which are hereby incorporated by reference in their entireties).

5.3. Libraries of Small Molecules

Libraries screened using the methods of the present invention can comprise a variety of types of test compounds. In some embodiments, the test compounds are nucleic acid or peptide molecules. In a non-limiting example, peptide molecules can exist in a phage display library. In other embodiments, types of test compounds include, but are not limited to, peptide analogs including peptides comprising non-naturally occurring amino acids, *e.g.*, D-amino acids, phosphorous analogs of amino acids, such as α-amino phosphoric acids and α-amino phosphoric acids, or amino acids having non-peptide linkages, nucleic acid analogs such as phosphorothioates and PNAs, hormones, antigens, synthetic or naturally occurring drugs, opiates, dopamine, serotonin, catecholamines, thrombin, acetylcholine, prostaglandins, organic molecules, pheromones, adenosine, sucrose, glucose, lactose and galactose. Libraries of polypeptides or proteins can also be used.

In a preferred embodiment, the combinatorial libraries are small organic molecule libraries, such as, but not limited to, benzodiazepines, isoprenoids, thiazolidinones, metathiazanones, pyrrolidines, morpholino compounds, and diazepindiones. In another embodiment, the combinatorial libraries comprise peptoids; random bio-oligomers; diversomers such as hydantoins, benzodiazepines and dipeptides; vinylogous polypeptides; nonpeptidal peptidomimetics; oligocarbamates; peptidyl phosphonates; peptide nucleic acid libraries; antibody libraries; or carbohydrate libraries.

Combinatorial libraries are themselves commercially available (see, *e.g.*, Advanced ChemTech Europe Ltd., Cambridgeshire, UK; ASINEX, Moscow Russia; BioFocus plc, Sittingbourne, UK; Bionet Research (A division of Key Organics Limited), Camelford, UK; ChemBridge Corporation, San Diego, California; ChemDiv Inc, San Diego, California.; ChemRx Advanced Technologies, South San Francisco, California; ComGenex Inc., Budapest, Hungary; Evotec OAI Ltd, Abingdon, UK; IF LAB Ltd., Kiev, Ukraine; Maybridge plc, Cornwall, UK; PharmaCore, Inc., North Carolina; SIDDCO Inc, Tucson, Arizona; TimTec Inc, Newark, Delaware; Tripos Receptor Research Ltd, Bude, UK; Toslab, Ekaterinburg, Russia).

In one embodiment, the combinatorial compound library for the methods of the present invention may be synthesized. There is a great interest in synthetic methods directed toward the creation of large collections of small organic compounds, or libraries, which could be screened for pharmacological, biological or other activity (Dolle, 2001, J. Comb. Chem. 3:477-517; Hall *et al.,* 2001, J. Comb. Chem. 3:125-150; Dolle, 2000, J. Comb. Chem. 2:383-433; Dolle, 1999, J. Comb. Chem. 1:235-282). The synthetic methods applied to create vast combinatorial libraries are performed in solution or in the solid phase, *i.e.*, on a solid support. Solid-phase synthesis makes it easier to conduct multi-step reactions and to drive reactions to completion with high yields because excess reagents can be easily added and washed away after each reaction step. Solid-phase combinatorial synthesis also tends to improve isolation, purification and screening. However, the more traditional solution phase chemistry supports a wider variety of organic reactions than solid-phase chemistry. Methods and strategies for the synthesis of combinatorial libraries can be found in *A Practical Guide to Combinatorial Chemistry,* A.W. Czarnik and S.H. Dewitt , eds., American Chemical Society, 1997; *The Combinatorial Index*, B.A. Bunin, Academic Press, 1998; *Organic Synthesis on Solid Phase*, F.Z. Dörwald, Wiley-VCH, 2000; and *Solid-Phase Organic Syntheses, Vol. 1*, A.W. Czarnik, ed., Wiley Interscience, 2001.

Combinatorial compound libraries of the present invention may be synthesized using apparatuses described in US Patent No. 6,358,479 to Frisina *et al.*, U.S. Patent No. 6,190,619 to Kilcoin *et al.*, US Patent No. 6,132,686 to Gallup *et al.*, US Patent No. 6,126,904 to Zuellig *et al.*, US Patent No. 6,074,613 to Harness *et al.*, US Patent No. 6,054,100 to Stanchfield *et al.*, and US Patent No. 5,746,982 to Saneii *et al.* which are hereby incorporated by reference in their entirety. These patents describe synthesis apparatuses capable of holding a plurality of reaction vessels for parallel synthesis of multiple discrete compounds or for combinatorial libraries of compounds.

In one embodiment, the combinatorial compound library can be synthesized in solution. The method disclosed in U.S. Patent No. 6,194,612 to Boger *et al.,* which is hereby incorporated by reference in its entirety, features compounds useful as templates for solution phase synthesis of combinatorial libraries. The template is designed to permit reaction products to be easily purified from unreacted reactants using liquid/liquid or solid/liquid extractions. The compounds produced by combinatorial synthesis using the template will preferably be small organic molecules. Some compounds in the library may mimic the effects of non-peptides or peptides. In contrast to solid phase synthesize of combinatorial compound libraries, liquid phase synthesis does not require the use of specialized protocols for monitoring the individual steps of a multistep solid phase synthesis (Egner *et al.*, 1995, J.Org. Chem. 60:2652; Anderson *et al.*, 1995, J. Org. Chem. 60:2650; Fitch *et al.*, 1994, J. Org. Chem. 59:7955; Look *et al.*, 1994, J. Org. Chem. 49:7588; Metzger *et al.*, 1993, Angew. Chem., Int. Ed. Engl. 32:894; Youngquist *et al.*, 1994, Rapid Commun. Mass Spect. 8:77; Chu *et al.*, 1995, J. Am. Chem. Soc. 117:5419; Brummel *et al.*, 1994, Science 264:399; Stevanovic *et al.*, 1993, Bioorg. Med. Chem. Lett. 3:431).

Combinatorial compound libraries useful for the methods of the present invention can be synthesized on solid supports. In one embodiment, a split synthesis method, a protocol of separating and mixing solid supports during the synthesis, is used to synthesize a library of compounds on solid supports (*see* Lam *et al.*, 1997, Chem. Rev. 97:41-448; Ohlmeyer *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926 and references cited therein). Each solid support in the final library has substantially one type of test compound attached to its surface. Other methods for synthesizing combinatorial libraries on solid supports, wherein one product is attached to each support, will be known to those of skill in the art (see, e.g., Nefzi et al., 1997, Chem. Rev. 97:449-472 and US Patent No. 6,087,186 to Cargill *et al.* which are hereby incorporated by reference in their entirety).

As used herein, the term "solid support" is not limited to a specific type of solid support. Rather a large number of supports are available and are known to one skilled in the art. Solid supports include silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads, polystyrene beads, alumina gels, and polysaccharides. A suitable solid support may be selected on the basis of desired end use and suitability for various synthetic protocols. For example, for peptide synthesis, a solid support can be a resin such as p-methylbenzhydrylamine (pMBHA) resin (Peptides International, Louisville, KY), polystyrenes (*e.g.*, PAM-resin obtained from Bachem Inc., Peninsula Laboratories, etc.), including chloromethylpolystyrene, hydroxymethylpolystyrene and

aminomethylpolystyrene, poly (dimethylacrylamide)-grafted styrene co-divinyl-benzene (*e.g.*, POLYHIPE resin, obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (*e.g.*, TENTAGEL or ARGOGEL, Bayer, Tubingen, Germany) polydimethylacrylamide resin (obtained from Milligen/Biosearch, California), or Sepharose (Pharmacia, Sweden).

In one embodiment, the solid phase support is suitable for *in vivo* use, *i.e.*, it can serve as a carrier or support for administration of the test compound to a patient (*e.g.*, TENTAGEL, Bayer, Tubingen, Germany). In a particular embodiment, the solid support is palatable and/or orally ingestable.

In some embodiments of the present invention, compounds can be attached to solid supports via linkers. Linkers can be integral and part of the solid support, or they may be nonintegral that are either synthesized on the solid support or attached thereto after synthesis. Linkers are useful not only for providing points of test compound attachment to the solid support, but also for allowing different groups of molecules to be cleaved from the solid support under different conditions, depending on the nature of the linker. For example, linkers can be, *inter alia*, electrophilically cleaved, nucleophilically cleaved, photocleavable, enzymatically cleaved, cleaved by metals, cleaved under reductive conditions or cleaved under oxidative conditions.

In another embodiment, the combinatorial compound libraries can be assembled *in situ* using dynamic combinatorial chemistry as described in European Patent Application 1,118,359 A1 to Lehn; Huc & Nguyen, 2001, Comb. Chem. High Throughput. Screen. 4:53-74; Lehn and Eliseev, 2001, Science 291:2331-2332; Cousins *et al.* 2000, Curr. Opin. Chem. Biol. 4: 270-279; and Karan & Miller, 2000, Drug. Disc. Today 5:67-75 which are incorporated by reference in their entirety.

Dynamic combinatorial chemistry uses non-covalent interaction with a target biomolecule, including but not limited to a protein, RNA, or DNA, to favor assembly of the most tightly binding molecule that is a combination of constituent subunits present as a mixture in the presence of the biomolecule. According to the laws of thermodynamics, when a collection of molecules is able to combine and recombine at equilibrium through reversible chemical reactions in solution, molecules, preferably one molecule, that bind most tightly to a templating biomolecule will be present in greater amount than all other possible combinations. The reversible chemical reactions include, but are not limited to, imine, acyl-hydrazone, amide, acetal, or ester formation between carbonyl-containing compounds and amines, hydrazines, or alcohols; thiol exchange between disulfides; alcohol

exchange in borate esters; Diels-Alder reactions; thermal- or photoinduced sigmatropic or electrocyclic rearrangements; or Michael reactions.

In the preferred embodiment of this technique, the constituent components of the dynamic combinatorial compound library are allowed to combine and reach equilibrium in the absence of the target RNA and then incubated in the presence of the target RNA, preferably at physiological conditions, until a second equilibrium is reached. The second, perturbed, equilibrium (the so-called "templated mixture") can, but need not necessarily, be fixed by a further chemical transformation, including but not limited to reduction, oxidation, hydrolysis, acidification, or basification, to prevent restoration of the original equilibrium when the dynamical combinatorial compound library is separated from the target RNA.

In the preferred embodiment of this technique, the predominant product or products of the templated dynamic combinatorial library can separated from the minor products and directly identified. In another embodiment, the identity of the predominant product or products can be identified by a deconvolution strategy involving preparation of derivative dynamic combinatorial libraries, as described in European Patent Application 1,118,359 A1, which is incorporated by reference in their entirety, whereby each component of the mixture is, preferably one-by-one but possibly group-wise, left out of the mixture and the ability of the derivative library mixture at chemical equilibrium to bind the target RNA is measured. The components whose removal most greatly reduces the ability of the derivative dynamic combinatorial library to bind the target RNA are likely the components of the predominant product or products in the original dynamic combinatorial library.

5.4. <u>Library Screening</u>

After a target nucleic acid, such as but not limited to RNA or DNA, is labeled and a test compound library is synthesized or purchased or both, the labeled target nucleic acid is used to screen the library to identify test compounds that bind to the nucleic acid. Screening comprises contacting a labeled target nucleic acid with an individual, or small group, of the components of the compound library. Preferably, the contacting occurs in an aqueous solution, and most preferably, under physiologic conditions. The aqueous solution preferably stabilizes the labeled target nucleic acid and prevents denaturation or degradation of the nucleic acid without interfering with binding of the test compounds. The aqueous solution can be similar to the solution in which a complex between the target RNA and its corresponding host cell factor (if known) is formed *in vitro*. For example, TK

buffer, which is commonly used to form Tat protein-TAR RNA complexes *in vitro*, can be used in the methods of the invention as an aqueous solution to screen a library of test compounds for TAR RNA binding compounds.

The methods of the present invention for screening a library of test compounds preferably comprise contacting a test compound with a target nucleic acid in the presence of an aqueous solution, the aqueous solution comprising a buffer and a combination of salts, preferably approximating or mimicking physiologic conditions. The aqueous solution optionally further comprises non-specific nucleic acids, such as, but not limited to, DNA; yeast tRNA; salmon sperm DNA; homoribopolymers such as, but not limited to, poly IC, polyA, polyU, and polyC; and non-specific RNA. The non-specific RNA may be an unlabeled target nucleic acid having a mutation at the binding site, which renders the unlabeled nucleic acid incapable of interacting with a test compound at that site. For example, if dye-labeled TAR RNA is used to screen a library, unlabeled TAR RNA having a mutation in the uracil 23/cytosine 24 bulge region may also be present in the aqueous solution. Without being bound by any theory, the addition of unlabeled RNA that is essentially identical to the dye-labeled target RNA except for a mutation at the binding site might minimize interactions of other regions of the dye-labeled target RNA with test compounds or with the solid support and prevent false positive results.

The solution further comprises a buffer, a combination of salts, and optionally, a detergent or a surfactant. The pH of the solution typically ranges from about 5 to about 8, preferably from about 6 to about 8, most preferably from about 6.5 to about 8. A variety of buffers may be used to achieve the desired pH. Suitable buffers include, but are not limited to, Tris, Mes, Bis-Tris, Ada, Aces, Pipes, Mopso, Bis-Tris propane, Bes, Mops, Tes, Hepes, Dipso, Mobs, Tapso, Trizma, Heppso, Popso, TEA, Epps, Tricine, Gly-Gly, Bicine, and sodium-potassium phosphate. The buffering agent comprises from about 10 mM to about 100 mM, preferably from about 25 mM to about 75 mM, most preferably from about 40 mM to about 60 mM buffering agent. The pH of the aqeuous solution can be optimized for different screening reactions, depending on the target RNA used and the types of test compounds in the library, and therefore, the type and amount of the buffer used in the solution can vary from screen to screen. In a preferred embodiment, the aqueous solution has a pH of about 7.4, which can be achieved using about 50 mM Tris buffer.

In addition to an appropriate buffer, the aqueous solution further comprises a combination of salts, from about 0 mM to about 100 mM KCl, from about 0 mM to about 1 M NaCl, and from about 0 mM to about 200 mM $MgCl_2$. In a preferred embodiment, the combination of salts is about 100 mM KCl, 500 mM NaCl, and 10 mM $MgCl_2$. Without

being bound by any theory, Applicant has found that a combination of KCl, NaCl, and $MgCl_2$ stabilizes the target RNA such that most of the RNA is not denatured or digested over the course of the screening reaction. The optional concentration of each salt used in the aqueous solution is dependent on the particular target RNA used and can be determined using routine experimentation.

The solution optionally comprises from about 0.01% to about 0.5% (w/v) of a detergent or a surfactant. Without being bound by any theory, a small amount of detergent or surfactant in the solution might reduce non-specific binding of the target RNA to the solid support and control aggregation and increase stability of target RNA molecules. Typical detergents useful in the methods of the present invention include, but are not limited to, anionic detergents, such as salts of deoxycholic acid, 1-heptanesulfonic acid, N-laurylsarcosine, lauryl sulfate, 1-octane sulfonic acid and taurocholic acid; cationic detergents such as benzalkonium chloride, cetylpyridinium, methylbenzethonium chloride, and decamethonium bromide; zwitterionic detergents such as CHAPS, CHAPSO, alkyl betaines, alkyl amidoalkyl betaines, N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, and phosphatidylcholine; and non-ionic detergents such as n-decyl a-D-glucopyranoside, n-decyl ß-D-maltopyranoside, n-dodecyl ß-D-maltoside, n-octyl ß-D-glucopyranoside, sorbitan esters, n-tetradecyl ß-D-maltoside, octylphenoxy polyethoxyethanol (Nonidet P-40), nonylphenoxypolyethoxyethanol (NP-40), and tritons. Preferably, the detergent, if present, is a nonionic detergent. Typical surfactants useful in the methods of the present invention include, but are not limited to, ammonium lauryl sulfate, polyethylene glycols, butyl glucoside, decyl glucoside, Polysorbate 80, lauric acid, myristic acid, palmitic acid, potassium palmitate, undecanoic acid, lauryl betaine, and lauryl alcohol. More preferably, the detergent, if present, is Triton X-100 and present in an amount of about 0.1% (w/v).

Non-specific binding of a labeled target nucleic acid to test compounds can be further minimized by treating the binding reaction with one or more blocking agents. In one embodiment, the binding reactions are treated with a blocking agent, *e.g.*, bovine serum albumin ("BSA"), before contacting with to the labeled target nucleic acid. In another embodiment, the binding reactions are treated sequentially with at least two different blocking agents. This blocking step is preferably performed at room temperature for from about 0.5 to about 3 hours. In a subsequent step, the reaction mixture is further treated with unlabeled RNA having a mutation at the binding site. This blocking step is preferably performed at about 4°C for from about 12 hours to about 36 hours before addition of the dye-labeled target RNA. Preferably, the solution used in the one or more blocking steps is

substantially similar to the aqueous solution used to screen the library with the dye-labeled target RNA, *e.g.*, in pH and salt concentration.

Once contacted, the mixture of labeled target nucleic acid and the test compound is preferably maintained at 4°C for from about 1 day to about 5 days, preferably from about 2 days to about 3 days with constant agitation. To identify the reactions in which binding to the labeled target nucleic acid occurred, after the incubation period, bound from free compounds are determined using an electrophoretic technique (see Section 5.5.1), or any of the methods disclosed in Section 5.5 *infra*. In another embodiment, the complexed target nucleic acid does not need to be separated from the free target nucleic acid if a technique (*i.e.*, spectrometry) that diferentiates between bound and unbound target nucleic acids is used.

The methods for identifying small molecules bound to labeled nucleic acid will vary with the type of label on the target nucleic acid. For example, if a target RNA is labeled with a visible of fluorescent dye, the target RNA complexes are preferably identified using a chromatographic technique that separates bound from free target by an electrophoretic or size differential technique using individual reactions. The reactions corresponding to changes in the migration of the complexed RNA can be cross-referenced to the small molecule compound(s) added to said reaction. Alternatively, complexed target RNA can be screened *en masse* and then separated from free target RNA using an electrophoretic or size differential technique, the resultant complexed target is then analyzed using a mass spectrometric technique. In this fashion the bound small molecule can be identified on the basis of its molecular weight. In this reaction *a priori* knowledge of the exact molecular weights of all compounds within the library is known. In another embodiment, the test compounds bound to the target nucleic acid may not require separation from the unbound target nucleic acid if a technique such as, but not limited to, spectrometry is used.

5.5. Separation Methods for Screening Test Compounds

Any method that detects an altered physical property of a target nucleic acid complexed to a test compound from the unbound target nucleic acid may be used for separation of the complexed and non-complexed target nucleic acids. Methods that can be utilized for the physical separation of complexed target RNA from unbound target RNA include, but are not limited to, electrophoresis, fluorescence spectroscopy, surface plasmon resonance, mass spectrometry, scintillation, proximity assay, structure-activity relationships ("SAR") by NMR spectroscopy, size exclusion chromatography, affinity chromatography,

and nanoparticle aggregation.

5.5.1. Electrophoresis

Methods for separation of the complex of a target RNA bound to a test compound from the unbound RNA comprises any method of electrophoretic separation, including but not limited to, denaturing and non-denaturing polyacrylamide gel electrophoresis, urea gel electrophoresis, gel filtration, pulsed field gel electrophoresis, two dimensional gel electrophoresis, continuous flow electrophoresis, zone electrophoresis, agarose gel electrophoresis, and capillary electrophoresis.

In a preferred embodiment, an automated electrophoretic system comprising a capillary cartridge having a plurality of capillary tubes is used for high-throughput screening of test compounds bound to target RNA. Such an apparatus for performing automated capillary gel electrophoresis is disclosed in U.S. Patent Nos. 5,885,430; 5,916,428; 6,027,627; and 6,063,251, the disclosures of which are incorporated by reference in their entireties.

The device disclosed in U.S. Patent No. 5,885,430, which is incorporated by reference in its entirety, allows one to simultaneously introduce samples into a plurality of capillary tubes directly from microtiter trays having a standard size. U.S. Patent No. 5,885,430 discloses a disposable capillary cartridge which can be cleaned between electrophoresis runs, the cartridge having a plurality of capillary tubes. A first end of each capillary tube is retained in a mounting plate, the first ends collectively forming an array in the mounting plate. The spacing between the first ends corresponds to the spacing between the centers of the wells of a microtiter tray having a standard size. Thus, the first ends of the capillary tubes can simultaneously be dipped into the samples present in the tray's wells. The cartridge is provided with a second mounting plate in which the second ends of the capillary tubes are retained. The second ends of the capillary tubes are arranged in an array which corresponds to the wells in the microtiter tray, which allows for each capillary tube to be isolated from its neighbors and therefore free from cross-contamination, as each end is dipped into an individual well.

Plate holes may be provided in each mounting plate and the capillary tubes inserted through these plate holes. In such a case, the plate holes are sealed airtight so that the side of the mounting plate having the exposed capillary ends can be pressurized. Application of a positive pressure in the vicinity of the capillary openings in this mounting plate allows for the introduction of air and fluids during electrophoretic operations and also can be used to force out gel and other materials from the capillary tubes during

reconditioning. The capillary tubes may be protected from damage using a needle comprising a cannula and/or plastic tubes, and the like when they are placed in these plate holes. When metallic cannula or the like are used, they can serve as electrical contacts for current flow during electrophoresis. In the presence of a second mounting plate, the second mounting plate is provided with plate holes through which the second ends of the capillary tubes project. In this instance, the second mounting plate serves as a pressure containment member of a pressure cell and the second ends of the capillary tubes communicate with an internal cavity of the pressure cell. The pressure cell is also formed with an inlet and an outlet. Gels, buffer solutions, cleaning agents, and the like may be introduced into the internal cavity through the inlet, and each of these can simultaneously enter the second ends of the capillaries.

In another preferred embodiment, the automated electrophoretic system can comprise a chip system consisting of complex designs of interconnected channels that perform and analyze enzyme reactions using part of a channel design as a tiny, continuously operating electrophoresis material, where reactions with one sample are going on in one area of the chip while electrophoretic separation of the products of another sample is taking place in a different part of the chip. Such a system is disclosed in U.S. Patent Nos. 5,699,157; 5,842,787; 5,869,004; 5,876,675; 5,942,443; 5,948,227; 6,042,709; 6,042,710; 6,046,056; 6,048,498; 6,086,740; 6,132,685; 6,150,119; 6,150,180; 6,153,073; 6,167,910; 6,171,850; and 6,186,660, the disclosures of which are incorporated by reference in their entireties.

The system disclosed in U.S. Patent No. 5,699,157, which is hereby incorporated by reference in its entirety, provides for a microfluidic system for high-speed electrophoretic analysis of subject materials for applications in the fields of chemistry, biochemistry, biotechnology, molecular biology and numerous other areas. The system has a channel in a substrate, a light source and a photoreceptor. The channel holds subject materials in solution in an electric field so that the materials move through the channel and separate into bands according to species. The light source excites fluorescent light in the species bands and the photoreceptor is arranged to receive the fluorescent light from the bands. The system further has a means for masking the channel so that the photoreceptor can receive the fluorescent light only at periodically spaced regions along the channel. The system also has an unit connected to analyze the modulation frequencies of light intensity received by the photoreceptor so that velocities of the bands along the channel are determined, which allows the materials to be analyzed.

The system disclosed in U.S. Patent No. 5,699,157 also provides for a

method of performing high-speed electrophoretic analysis of subject materials, which comprises the steps of holding the subject materials in solution in a channel of a microfluidic system; subjecting the materials to an electric field so that the subject materials move through the channel and separate into species bands; directing light toward the channel; receiving light from periodically spaced regions along the channel simultaneously; and analyzing the frequencies of light intensity of the received light so that velocities of the bands along the channel can be determined for analysis of said materials. The determination of the velocity of a species band determines the electrophoretic mobility of the species and its identification.

U.S. Patent No. 5,842,787, which is hereby incorporated by reference in its entirety, is generally directed to devices and systems employ channels having, at least in part, depths that are varied over those which have been previously described (such as the device disclosed in U.S. Patent No. 5,699,157), wherein said channel depths provide numerous beneficial and unexpected results such as but not limited to, a reduction in sample perturbation, reduced non-specific sample mixture by diffusion, and increased resolution.

In another embodiment, the electrophoretic method of separation comprises polyacrylamide gel electrophoresis. In a preferred embodiment, the polyacrylamide gel electrophoresis is non-denaturing, so as to differentiate the mobilities of the target RNA bound to a test compound from free target RNA. If the polyacrylamide gel electrophoresis is denaturing, then the target RNA:test compound complex must be cross-linked prior to electrophoresis to prevent the disassociation of the target RNA from the test compound during electrophoresis. Such techniques are well known to one of skill in the art.

In one embodiment of the method, the binding of test compounds to target nucleic acid can be detected, preferably in an automated fashion, by gel electrophoretic analysis of interference footprinting. RNA can be degraded at specific base sites by enzymatic methods such as ribonucleases A, U_2, CL_3, T_1, Phy M, and *B. cereus* or chemical methods such as diethylpyrocarbonate, sodium hydroxide, hydrazine, piperidine formate, dimethyl sulfate, [2,12-dimethyl-3,7,11,17-tetraazacyclo[11.3.1]heptadeca-1(17),2,11,13,15-pentaenato] nickel(II) (NiCR), cobalt(II)chloride, or iron(II) ethylenediaminetetraacetate (Fe-EDTA) as described for example in Zheng *et al.*, 1999, Biochem. 37:2207-2214; Latham & Cech, 1989, Science 245:276-282; and Sambrook *et al.*, 2001, in Molecular Cloning: A Laboratory Manual, pp 12.61-12.73, Cold Spring Harbor Laboratory Press, and the references cited therein, which are hereby incorporated by reference in their entireties. The

specific pattern of cleavage sites is determined by the accessibility of particular bases to the reagent employed to initiate cleavage and, as such, is therefore is determined by the three-dimensional structure of the RNA.

The interaction of small molecules with a target nucleic acid can change the accessibility of bases to these cleavage reagents both by causing conformational changes in the target nucleic acid or by covering a base at the binding interface. When a test compound binds to the nucleic acid and changes the accessibility of bases to cleavage reagents, the observed cleavage pattern will change. This method can be used to identify and characterize the binding of small molecules to RNA as described, for example, by Prudent *et al.,* 1995, J. Am. Chem. Soc. 117:10145-10146 and Mei *et al.*, 1998, Biochem. 37:14204-14212.

In the preferred embodiment of this technique, the detectably labeled target nucleic acid is incubated with an individual test compound and then subjected to treatment with a cleavage reagent, either enzymatic or chemical. The reaction mixture can be preferably be examined directly, or treated further to isolate and concentrate the nucleic acid. The fragments produced are separated by electrophoresis and the pattern of cleavage can be compared to a cleavage reaction performed in the absence of test compound. A change in the cleavage pattern directly indicates that the test compound binds to the target nucleic acid. Multiple test compounds can be examined both in parallel and serially.

Other embodiments of electrophoretic separation include, but are not limited to urea gel electrophoresis, gel filtration, pulsed field gel electrophoresis, two dimensional gel electrophoresis, continuous flow electrophoresis, zone electrophoresis, and agarose gel electrophoresis.

5.5.2. Fluorescence Spectroscopy

In a preferred embodiment, fluorescence polarization spectroscopy, an optical detection method that can differentiate the proportion of a fluorescent molecule that is either bound or unbound in solution (*e.g.*, the labeled target nucleic acid of the present invention), can be used to read reaction results without electrophoretic separation of the samples. Fluorescence polarization spectroscopy can be used to read the reaction results in the chip system disclosed in U.S. Patent Nos. 5,699,157; 5,842,787; 5,869,004; 5,876,675; 5,942,443; 5,948,227; 6,042,709; 6,042,710; 6,046,056; 6,048,498; 6,086,740; 6,132,685; 6,150,119; 6,150,180; 6,153,073; 6,167,910; 6,171,850; and 6,186,660, the disclosures of which are incorporated by reference in their entireties. The application of fluorescence

polarization spectroscopy to the chip system disclosed in the U.S. Patents listed *supra* is fast, efficient, and well-adapted for high-throughput screening.

In another embodiment, a compound that has an affinity for the target nucleic acid of interest can be labeled with a fluorophore to screen for test compounds that bind to the target nucleic acid. For example, a pyrene-containing aminoglycoside analog was used to accurately monitor antagonist binding to a prokaryotic 16S rRNA A site (which comprises the natural target for aminoglycoside antibiotics) in a screen using a fluorescence quenching technique in a 96-well plate format (Hamasaki & Rando, 1998, Anal. Biochem. 261(2):183-90).

In another embodiment, fluorescence resonance energy transfer (FRET) can be used to screen for test compounds that bind to the target nucleic acid. FRET, a characteristic change in fluorescence, occurs when two fluorophores with overlapping emission and excitation wavelength bands are held together in close proximity, such as by a binding event. In the preferred embodiment, the fluorophore on the target nucleic acid and the fluorophore on the test compounds will have overlapping excitation and emission spectra such that one fluorophore (the donor) transfers its emission energy to excite the other fluorophore (the acceptor). The acceptor preferably emits light of a different wavelength upon relaxing to the ground state, or relaxes non-radiatively to quench fluorescence. FRET is very sensitive to the distance between the two fluorophores, and allows measurement of molecular distances less than 10 nm. For example, U.S, Patent 6,337,183 to Arenas *et al.*, which is incorporated by reference in its entirety, describes a screen for compounds that bind RNA that uses FRET to measure the effect of test compounds on the stability of a target RNA molecule where the target RNA is labeled with both fluorescent acceptor and donor molecules and the distance between the two fluorophores as determined by FRET provides a measure of the folded structure of the RNA. Matsumoto *et al.* (2000, Bioorg. Med. Chem. Lett. 10:1857-1861) describe a system where a peptide that binds to HIV-1 TAR RNA is labeled on one end with a fluorescein fluorophore and a tetramethylrhodamine on the other end. The conformational change of the peptide upon binding to the RNA provided a FRET signal to screen for compounds that bound to the TAR RNA.

In the preferred embodiment, both the target nucleic acid and a compound that has an affinity for the target nucleic acid of interest are labeled with fluorophores with overlapping emission and excitation spectra (donor and acceptor), including but not limited to fluorescein and derivatives, rhodamine and derivatives, cyanine dyes and derivatives, bora-3a,4a-diaza-s-indacene (BODIPY®) and derivatives, pyrene, nanoparticles, or

non-fluorescent quenching molecules. Binding of a labeled test compound to the target nucleic acid can be identified by the change in observable fluorescence as a result of FRET.

If the target nucleic acid is labeled with the donor fluorophore, then the test compounds is labeled with the acceptor fluorophore. Conversely, if the target nucleic acid is labeled with the acceptor fluorophore, then the test compounds is labeled with the donor fluorophore. A wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions. The fluorophore on the target nucleic acid must be in close proximity to the binding site of the test compounds, but should not be incorporated into a target nucleic acid at the specific binding site at which test compounds are likely to bind, since the presence of a covalently attached label might interfere sterically or chemically with the binding of the test compounds at this site.

In yet another embodiment, homogeneous time-resolved fluorescence ("HTRF") techniques based on time-resolved energy transfer from lanthanide ion complexes to a suitable acceptor species can be adapted for high-throughput screening for inhibitors of RNA-protein complexes (Hemmilä, 1999, J. Biomol. Screening 4:303-307; Mathis, 1999, J. Biomol. Screening 4:309-313). HTRF is similar to fluorescence resonance energy transfer using conventional organic dye pairs, but has several advantages, such as increased sensitivity and efficiency, and background elimination (Xavier *et al.*, 2000, Trends Biotechnol. 18(8):349-356).

Fluorescence spectroscopy has traditionally been used to characterize DNA-protein and protein-protein interactions, but fluorescence spectroscopy has not been widely used to characterize RNA-protein interactions because of an interfering absorption of RNA nucleotides with the intrinsic tryptophan fluorescence of proteins (Xavier *et al.*, 2000, Trends Biotechnol. 18(8):349-356.). However, fluorescence spectroscopy has been used in studying the single tryptophan residue within the arginine-rich RNA-binding domain of Rev protein and its interaction with the RRE in a time-resolved fluorescence study (Kwon & Carson, 1998, Anal. Biochem. 264:133-140). Thus, in this invention, fluorescence spectroscopy is less preferred if the test compounds or peptides or proteins possess intrinsic tryptophan fluorescence. However, fluorescence spectroscopy can be used for test compounds that do not possess intrinsic fluorescence.

5.5.3. Surface Plasmon Resonance ("SPR")

Surface plasmon resonance (SPR) can be used for determining kinetic rate constants and equilibrium constants for macromolecular interactions by following the

association project in "real time" (Schuck, 1997, Annu. Rev. Biophys. Biomol. Struct. 26:541-566).

The principle of SPR is summarized by Xavier *et al.* (Trends Biotechnol., 2000, 18(8):349-356) as follows. Total internal reflection occurs at the boundary between two substances of different refractive index. The incident light's electromagnetic field penetrates beyond the interface as an evanescent wave, which extends a few hundred nanometers beyond the surface into the medium. Insertion of a thin gold foil at the interace produced SPR owing to the absorption of the energy from the evanescent wave by free electron clouds of the metal (plasmons). As a result of this absorbance, there is a drop in the intensity of the reflected light at a particular angle of incidence. The evanescent wave profile depends exquisitely on the refractive index of the medium it probes. Thus, the angle at which absorption occurs is very sensitive to the refractive changes in the external medium. All proteins and nucleic acids are known to change the refractive index of water by a similar amount per unit mass, irrespective of their amino acid or nucleotide composition (the refractive index change is different for proteins and nucleic acids). When the protein or nucleic acid content of the layer at the sensor changes, the refractive index also changes. Typically, one member of a complex is immobilized in a dextran layer and then the other member is introduced into the solution, either in a flow cell (Biacore AB, Uppsala, Sweden) or a stirred cuvette (Affinity Sensors, Santa Fe, New Mexico). It has been determined that there is a linear correlation between the surface concentration of protein or nucleic acid and the shift in resonance angle, which can be used to quantitate kinetic rate constants and/or the equilibrium constants.

In the present invention, the target RNA may be immobilized to the sensor surface through a streptavidin-biotin linkage, the linkage of which is disclosed by Crouch *et al.* (Methods Mol. Biol., 1999, 118:143-160). The RNA is biotinylated either during synthesis or post-synthetically via the conversion of the 3' terminal ribonucleoside of the RNA into a reactive free amino group or using a T7 polymerase incorporated guanosine monophosphorothioate at the 5' end. SPR has been used to determine the stoichiometry and affinity of the interaction between the HIV Rev protein and the RRE (Van Ryk & Venkatesan, 1999, J. Biol. Chem. 274:17452-17463) and the aminoglycoside antibiotics with RRE and a model RNA derived from the 16S ribosomal A site, respectively (Hendrix *et al.*, 1997, J. Am. Chem. Soc. 119:3641-3648; Wong *et al.*, 1998, Chem. Biol. 5:397-406).

In one embodiment of the present invention, the target nucleic aeid can be immobilized to a sensor surface (*e.g.*, by a streptavidin-biotin linkage) and SPR can be used

to (a) determine whether the target RNA binds a test compound and (b) further characterize the binding of the target nucleic acids of the present invention to a test compound.

5.5.4. Mass Spectrometry

An automated method for analyzing mass spectrometer data which can analyze complex mixtures containing many thousands of components and can correct for background noise, multiply charged peaks and atomic isotope peaks is described in U.S. Patent No. 6,147,344, which is hereby incorporated by reference in its entirety. The system disclosed in U.S. Patent No. 6,147,344 is a method for analyzing mass spectrometer data in which a control sample measurement is performed providing a background noise check. The peak height and width values at each m/z ratio as a function of time are stored in a memory. A mass spectrometer operation on a material to be analyzed is performed and the peak height and width values at each m/z ratio versus time are stored in a second memory location. The mass spectrometer operation on the material to be analyzed is repeated a fixed number of times and the stored control sample values at each m/z ratio level at each time increment are subtracted from each corresponding one from the operational runs, thus producing a difference value at each mass ratio for each of the multiple runs at each time increment. If the MS value minus the background noise does not exceed a preset value, the m/z ratio data point is not recorded, thus eliminating background noise, chemical noise and false positive peaks from the mass spectrometer data. The stored data for each of the multiple runs is then compared to a predetermined value at each m/z ratio and the resultant series of peaks, which are now determined to be above the background, is stored in the m/z points in which the peaks are of significance.

One possibility for the utilization of mass spectrometry in high throughput screening is the integration of SPR with mass spectrometry. Approaches that have been tried are direct analysis of the analyte retained on the sensor chip and mass spectrometry with the eluted analyte (Sonksen *et al.*, 1998, Anal. Chem. 70:2731-2736; Nelson & Krone, 1999, J. Mol. Recog. 12:77-93). Further developments, especially in the interfacing of the sensor chip with the mass spectrometer and in reusing the sensor chip, are required to make SPR combined with mass spectroscopy a high-throughput method for biomolecular interaction analysis and the screening of targets for small molecule inhibitors (Xavier *et al.*, 2000, Trends Biotechnol. 18(8):349-356).

In one embodiment of the present invention, the target nucleic acid complexed to a test compound can be determined by any of the mass spectrometry processed described *supra*. Furthermore, mass spectrometry can also be used to elucidate

the structure of the test compound.

5.5.5. Scintillation Proximity Assay ("SPA")

Scintillation Proximity Assay ("SPA") is a method that can be used for screening small molecules that bind to the target RNAs. SPA would involve radiolabeling either the target RNA or the test compound and then quantitating its binding to the other member to a bead or a surface impregnated with a scintillant (Cook, 1996, Drug Discov. Today 1:287-294). Currently, fluorescence-based techniques are preferred for high-throughput screening (Pope *et al.*, 1999, Drug Discov. Today 4:350-362).

Screening for small molecules that inhibit Tat peptide:TAR RNA interaction has been performed with SPA, and inhibitors of the interaction were isolated and characterized (Mei *et al.*, 1997, Bioorg. Med. Chem. 5:1173-1184; Mei *et al.*, 1998, Biochemistry 37:14204-14212). A similar approach can be used to identify small molecules that directly bind to a preselected target RNA element in accordance with the invention can be utilized.

SPA can be adapted to high throughput screening by the availability of microplates, wherein the scintillant is directly incorporated into the plastic of the microtiter wells (Nakayama *et al.*, 1998, J. Biomol. Screening 3:43-48). Thus, one embodiment of the present invention comprises (a) labeling of the target nucleic acid with a radioactive or fluorescent label; (b) contacted the labeled nucleic acid with test compounds, wherein each test compound is in a microtiter well coated with scintillant and is tethered to the microtiter well; and (c) identifying and quantifying the test compounds bound to the target nucleic acid with SPA, wherein the test compound is identified by virtue of its location in the microplate.

5.5.6. Structure-Activity Relationships ("SAR") by NMR Spectroscopy

NMR spectroscopy is a valuable technique for identifying complexed target nucleic acids by qualitatively determining changes in chemical shift, specifically from distances measured using relaxation effects, and NMR-based approaches have been used in the identification of small molecule binders of protein drug targets (Xavier *et al.*, 2000, Trends Biotechnol. 18(8):349-356). The determination of structure-activity relationships ("SAR") by NMR is the first method for NMR described in which small molecules that bind adjacent subsites are identified by two-dimentional ^{1}H-^{15}N spectra of the target protein (Shuker *et al.*, 1996, Science 274:1531-1534). The signal from the bound molecule is monitored by employing line broadening, transferred NOEs and pulsed field gradient

diffusion measurements (Moore, 1999, Curr. Opin. Biotechnol. 10:54-58). A strategy for lead generation by NMR using a library of small molecules has been recently described (Fejzo *et al.*, 1999, Chem. Biol. 6:755-769).

In one embodiment of the present invention, the target nucleic acid complexed to a test compound can be determined by SAR by NMR. Furthermore, SAR by NMR can also be used to elucidate the structure of the test compound.

5.5.7. Size Exclusion Chromatography

In another embodiment of the present invention, size-exclusion chromatography is used to purify test compounds that are bound to a target nucleic from a complex mixture of compounds. Size-exclusion chromatography separates molecules based on their size and uses gel-based media comprised of beads with specific size distributions. When applied to a column, this media settles into a tightly packed matrix and forms a complex array of pores. Separation is accomplished by the inclusion or exclusion of molecules by these pores based on molecular size. Small molecules are included into the pores and, consequently, their migration through the matrix is retarded due to the added distance they must travel before elution. Large molecules are excluded from the pores and migrate with the void volume when applied to the matrix. In the present invention, a target nucleic acid is incubated with a mixture of test compounds while free in solution and allowed to reach equilibrium. When applied to a size exclusion column, test compounds free in solution are retained by the column, and test compounds bound to the target nucleic acid are passed through the column. In a preferred embodiment, spin columns commonly used for "desalting" of nucleic acids will be employed to separate bound from unbound test compounds (*e.g.*, Bio-Spin columns manufactured by BIO-RAD). In another embodiment, the size exclusion matrix is packed into multiwell plates to allow high throughput separation of mixtures (*e.g.*, PLASMID 96-well SEC plates manufactured by Millipore).

5.5.8. Affinity Chromatography

In one embodiment of the present invention, affinity capture is used to purify test compounds that are bound to a target nucleic acid labeled with an affinity tag from a complex mixture of compounds. To accomplish this, a target nucleic acid labeled with an affinity tag is incubated with a mixture of test compounds while free in solution and then captured to a solid support once equilibrium has been established; alternatively, target nucleic acids labeled with an affinity tag can be captured to a solid support first and then allowed to reach equilibrium with a mixture of test compounds.

The solid support is typically comprised of, but not limited to, cross-linked agarose beads that are coupled with a ligand for the affinity tag. Alternatively, the solid support may be a glass, silicon, metal, or carbon, plastic (polystyrene, polypropylene) surface with or without a self-assembled monolayer (SAM) either with a covalently attached ligand for the affinity tag, or with inherent affinity for the tag on the target nucleic acid.

Once the complex between the target nucleic acid and test compound has reached equilibrium and has been captured, one skilled in the art will appreciate that the retention of bound compounds and removal of unbound compounds is facilitated by washing the solid support with large excesses of binding reaction buffer. Furthermore, retention of high affinity compounds and removal of low affinity compounds can be accomplished by a number of means that increase the stringency of washing; these means include, but are not limited to, increasing the number and duration of washes, raising the salt concentration of the wash buffer, addition of detergent or surfactant to the wash buffer, and addition of non-specific competitor to the wash buffer.

In one embodiment, the test compounds themselves are detectably labeled with fluorescent dyes, radioactive isotopes, or nanoparticles. When the test compounds are applied to the captured target nucleic acid in a spatially addressed fashion (*e.g.*, in separate wells of a 96-well microplate), binding between the test compounds and the target nucleic acid can be determined by the presence of the detectable label on the test compound using fluorescence.

Following the removal of unbound compounds, bound compounds with high affinity for the target nucleic acid can be eluted from the immobilized target nucleic acids and analyzed. The elution of test compounds can be accomplished by any means that break the non-covalent interactions between the target nucleic acid and compound. Means for elution include, but are not limited to, changing the pH, changing the salt concentration, the application of organic solvents, and the application of molecules that compete with the bound ligand. In a preferred embodiment, the means employed for elution will release the compound from the target RNA, but will not effect the interaction between the affinity tag and the solid support, thereby achieving selective elution of test compound. Moreover, a preferred embodiment will employ an elution buffer that is volatile to allow for subsequent concentration by lyophilization of the eluted compound (e.g., 0 M to 5 M ammonium acetate).

5.5.9. Nanoparticle Aggregation

In one embodiment of the present invention, both the target nucleic acid and the test compounds are labeled with nanoparticles. A nanoparticle is a cluster of ions with controlled size from 0.1 to 1000 nm comprised of metals, metal oxides, or semiconductors including, but not limited to Ag_2S, ZnS, CdS, CdTe, Au, or TiO_2. Methods for the attachment of nucleic acids and small molecules to nanoparticles are well know to one of skill in the art (reviewed in Niemeyer, 2001, Angew. Chem. Int. Ed. 40:4129-4158. The references cited therein are hereby incorporated by reference in their entireties). In particular, if multiple copies of the target nucleic acid are attached to a single nanoparticle and multiple copies of a test compound are attached to another nanoparticle, then interaction between the test compound and target nucleic acid will induce aggregation of nanoparticles as described, for example, by Mitchel *et al.* 1999, J. Am. Chem. Soc. 121:8122-8123. The aggregate can be detected by changes in absorbance or fluorescence spectra and physically separated from the unbound components through filtration or centrifugation.

5.6. Methods for Identifying or Characterizing the Test Compounds Bound to the Target Nucleic Acids

If the library comprises arrays or microarrays of test compounds, wherein each test compound has an address or identifier, the test compound can be deconvoluted, *e.g.*, by cross-referencing the positive sample to original compound list that was applied to the individual test assays.

If the library is a peptide or nucleic acid library, the sequence of the test compound can be determined by direct sequencing of the peptide or nucleic acid. Such methods are well known to one of skill in the art.

A number of physico-chemical techniques can be used for the de novo characterization of test compounds bound to the target.

5.6.1. Mass Spectrometry

Mass spectrometry (*e.g.,* electrospray ionization ("ESI") and matrix-assisted laser desorption-ionization ("MALDI"), Fourier-transform ion cyclotron resonance ("FT-ICR")) can be used both for high-throughput screening of test compounds that bind to a target RNA and elucidating the structure of the test compound. Thus, one example of mass spectroscopy is that separation of a bound and unbound complex and test compound structure elucidation can be carried out in a single step.

MALDI uses a pulsed laser for desorption of the ions and a time-of-flight analyzer, and has been used for the detection of noncovalent tRNA:amino-acyl-tRNA synthetase complexes (Gruic-Sovulj *et al.*, 1997, J. Biol. Chem. 272:32084-32091). However, covalent cross-linking between the target nucleic acid and the test compound is required for detection, since a non-covalently bound complex may dissociate during the MALDI process.

ESI mass spectrometry ("ESI-MS") has been of greater utility for studying non-covalent molecular interactions because, unlike the MALDI process, ESI-MS generates molecular ions with little to no fragmentation (Xavier *et al.*, 2000, Trends Biotechnol. 18(8):349-356). ESI-MS has been used to study the complexes formed by HIV Tat peptide and protein with the TAR RNA (Sannes-Lowery *et al.*, 1997, Anal. Chem. 69:5130-5135).

Fourier-transform ion cyclotron resonance ("FT-ICR") mass spectrometry provides high-resolution spectra, isotope-resolved precursor ion selection, and accurate mass assignments (Xavier *et al.*, 2000, Trends Biotechnol. 18(8):349-356). FT-ICR has been used to study the interaction of aminoglycoside antibiotics with cognate and non-cognate RNAs (Hofstadler *et al.*, 1999, Anal. Chem. 71:3436-3440; Griffey *et al.*, 1999, Proc. Natl. Acad. Sci. USA 96:10129-10133). As true for all of the mass spectrometry methods discussed herein, FT-ICR does not require labeling of the target RNA or a test compound.

An advantage of mass spectroscopy is not only the elucidation of the structure of the test compound, but also the determination of the structure of the test compound bound to the preselected target RNA. Such information can enable the discovery of a consensus structure of a test compound that specifically binds to a preselected target RNA.

5.6.2. NMR Spectroscopy

As described above, NMR spectroscopy is a technique for identifying binding sites in target nucleic acids by qualitatively determining changes in chemical shift, specifically from distances measured using relaxation effects. Examples of NMR that can be used for the invention include, but are not limited to, one-dimentional NMR, two-dimentional NMR, correlation spectroscopy ("COSY"), and nuclear Overhauser effect ("NOE") spectroscopy. Such methods of structure determination of test compounds are well known to one of skill in the art.

Similar to mass spectroscopy, an advantage of NMR is the not only the elucidation of the structure of the test compound, but also the determination of the structure

of the test compound bound to the preselected target RNA. Such information can enable the discovery of a consensus structure of a test compound that specifically binds to a preselected target RNA.

5.6.3. Vibrational Spectroscopy

Vibrational spectroscopy (*e.g.* infrared (IR) spectroscopy or Raman spectroscopy) can be used for elucidating the structure of the test compound on the isolated bead.

Infrared spectroscopy measures the frequencies of infrared light (wavelengths from 100 to 10,000 nm) absorbed by the test compound as a result of excitation of vibrational modes according to quantum mechanical selection rules which require that absorption of light cause a change in the electric dipole moment of the molecule. The infrared spectrum of any molecule is a unique pattern of absorption wavelengths of varying intensity that can be considered as a molecular fingerprint to identify any compound.

Infrared spectra can be measured in a scanning mode by measuring the absorption of individual frequencies of light, produced by a grating which separates frequencies from a mixed-frequency infrared light source, by the test compound relative to a standard intensity (double-beam instrument) or pre-measured ('blank') intensity (single-beam instrument). In a preferred embodiment, infrared spectra are measured in a pulsed mode (FT-IR) where a mixed beam, produced by an interferometer, of all infrared light frequencies is passed through or reflected off the test compound. The resulting interferogram, which may or may not be added with the resulting interferograms from subsequent pulses to increase the signal strength while averaging random noise in the electronic signal, is mathematically transformed into a spectrum using Fourier Transform or Fast Fourier Transform algorithms.

Raman spectroscopy measures the difference in frequency due to absorption of infrared frequencies of scattered visible or ultraviolet light relative to the incident beam. The incident monochromatic light beam, usually a single laser frequency, is not truly absorbed by the test compound but interacts with the electric field transiently. Most of the light scattered off the sample with be unchanged (Rayleigh scattering) but a portion of the scatter light will have frequencies that are the sum or difference of the incident and molecular vibrational frequencies. The selection rules for Raman (inelastic) scattering require a change in polarizability of the molecule. While some vibrational transitions are observable in both infrared and Raman spectrometry, must are observable only with one or

the other technique. The Raman spectrum of any molecule is a unique pattern of absorption wavelengths of varying intensity that can be considered as a molecular fingerprint to identify any compound.

Raman spectra are measured by submitting monochromatic light to the sample, either passed through or preferably reflected off, filtering the Rayleigh scattered light, and detecting the frequency of the Raman scattered light. An improved Raman spectrometer is described in US Patent No. 5,786,893 to Fink *et al.*, which is hereby incorporated by reference.

Vibrational microscopy can be measured in a spatially resolved fashion to address single beads by integration of a visible microscope and spectrometer. A microscopic infrared spectrometer is described in U.S. Patent No. 5,581,085 to Reffner *et al.*, which is hereby incorporated by reference in its entirety. An instrument that simultaneously performs a microscopic infrared and microscopic Raman analysis on a sample is described in U.S. Patent No. 5,841,139 to Sostek *et al.*, which is hereby incorporated by reference in its entirety.

In the preferred embodiment, test compounds can be identified by matching the IR or Raman spectra of a test compound to a dataset of vibrational (IR or Raman) spectra previously acquired for each compound in the combinatorial library. By this method, the spectra of compounds with known structure are recorded so that comparison with these spectra can identify compounds again when isolated from RNA binding experiments.

5.7. Secondary Biological Screens

The test compounds identified in the binding assay (for convenience referred to herein as a "lead" compound) can be tested for biological activity using host cells containing or engineered to contain the target RNA element coupled to a functional readout system. For example, the lead compound can be tested in a host cell engineered to contain the target RNA element controlling the expression of a reporter gene. In this example, the lead compounds are assayed in the presence or absence of the target RNA. Alternatively, a phenotypic or physiological readout can be used to assess activity of the target RNA in the presence and absence of the lead compound.

In one embodiment, the lead compound can be tested in a host cell engineered to contain the target RNA element controlling the expression of a reporter gene, such as, but not limited to, β-galactosidase, green fluorescent protein, red fluoreseent protein, luciferase, chloramphenicol acetyltransferase, alkaline phosphatase, and β-

lactamase. In a preferred embodiment, a cDNA encoding the target element is fused upstream to a reporter gene wherein translation of the reporter gene is repressed upon binding of the lead compound to the target RNA. In other words, the steric hindrance caused by the binding of the lead compound to the target RNA repressed the translation of the reporter gene. This method, termed the translational repression assay procedure ("TRAP") has been demonstrated in *E. coli* and *S. cerevisiae* (Jain & Belasco, 1996, Cell 87(1):115-25; Huang & Schreiber, 1997, Proc. Natl. Acad. Sci. USA 94:13396-13401).

In another embodiment, a phenotypic or physiological readout can be used to assess activity of the target RNA in the presence and absence of the lead compound. For example, the target RNA may be overexpressed in a cell in which the target RNA is endogenously expressed. Where the target RNA controls expression of a gene product involved in cell growth or viability, the *in vivo* effect of the lead compound can be assayed by measuring the cell growth or viability of the target cell. Alternatively, a reporter gene can also be fused downstream of the target RNA sequence and the effect of the lead compound on reporter gene expression can be assayed.

Alternatively, the lead compounds identified in the binding assay can be tested for biological activity using animal models for a disease, condition, or syndrome of interest. These include animals engineered to contain the target RNA element coupled to a functional readout system, such as a transgenic mouse. Animal model systems can also be used to demonstrate safety and efficacy.

Compounds displaying the desired biological activity can be considered to be lead compounds, and will be used in the design of congeners or analogs possessing useful pharmacological activity and physiological profiles. Following the identification of a lead compound, molecular modeling techniques can be employed, which have proven to be useful in conjunction with synthetic efforts, to design variants of the lead that can be more effective. These applications may include, but are not limited to, Pharmacophore Modeling (*cf.* Lamothe, *et al.* 1997, J. Med. Chem. 40: 3542; Mottola *et al.* 1996, J. Med. Chem. 39: 285; Beusen *et al.* 1995, Biopolymers 36: 181; P. Fossa *et al.* 1998, Comput. Aided Mol. Des. 12: 361), QSAR development (*cf.* Siddiqui *et al.* 1999, J. Med. Chem. 42: 4122; Barreca *et al.* 1999 Bioorg. Med. Chem. 7: 2283; Kroemer *et al.* 1995, J. Med. Chem. 38: 4917; Schaal *et al.* 2001, J. Med. Chem. 44: 155; Buolamwini & Assefa 2002, J. Mol. Chem. 45: 84), Virtual docking and screening/scoring (*cf.* Anzini *et al.* 2001, J. Med. Chem. 44: 1134; Faaland *et al.* 2000, Biochem. Cell. Biol. 78: 415; Silvestri *et al.* 2000, Bioorg. Med. Chem. 8: 2305; J. Lee *et al.* 2001, Bioorg. Med. Chem. 9: 19), and Structure Prediction using RNA structural programs including, but not limited to mFold (as described

by Zuker *et al.* Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide in RNA Biochemistry and Biotechnology pp. 11-43, J. Barciszewski & B.F.C. Clark, eds. (NATO ASI Series, Kluwer Academic Publishers, 1999) and Mathews *et al.* 1999 J. Mol. Biol. 288: 911-940); RNAmotif (Macke *et al.* 2001, Nucleic Acids Res. 29: 4724-4735; and the Vienna RNA package (Hofacker *et al.* 1994, Monatsh. Chem. 125: 167-188).

Further examples of the application of such techniques can be found in several review articles, such as Rotivinen *et al.*, 1988, Acta Pharmaceutical Fennica 97:159-166; Ripka, 1998, New Scientist 54-57; McKinaly & Rossmann, 1989, Annu. Rev. Pharmacol. Toxiciol. 29:111-122; Perry & Davies, QSAR: Quantitative Structure-Activity Relationships in Drug Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis & Dean, 1989, Proc. R. Soc. Lond. 236:125-140 and 141-162; Askew et al., 1989, J. Am. Chem. Soc. 111:1082-1090. Molecular modeling tools employed may include those from Tripos, Inc., St. Louis, Missouri (*e.g.*, Sybyl/UNITY, CONCORD, DiverseSolutions), Accelerys, San Diego, California (*e.g.*, Catalyst, Wisconsin Package {BLAST, etc.}), Schrodinger, Portland, Oregon (*e.g.*, QikProp, QikFit, Jaguar) or other such vendors as BioDesign, Inc. (Pasadena, California), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Cambridge, Ontario, Canada), and may include privately designed and/or "academic" software (*e.g.* RNAMotif, mFOLD). These application suites and programs include tools for the atomistic construction and analysis of structural models for drug-like molecules, proteins, and DNA or RNA and their potential interactions. They also provide for the calculation of important physical properties, such as solubility estimates, permeability metrics, and empirical measures of molecular "druggability" (*e.g.*, Lipinski "Rule of 5" as described by Lipinski *et al.* 1997, Adv. Drug Delivery Rev. 23: 3-25). Most importantly, they provide appropriate metrics and statistical modeling power (such as the patented CoMFA technology in Sybyl as described in US Patents 6,240,374 and 6,185,506) to develop Quantitative Structural Activity Relationships (QSARs) which are used to guide the synthesis of more efficacious clinical development candidates while improving desirable physical properties, as determined by results from the aforementioned secondary screening protocols.

5.8. Use of Identified Compounds That Bind RNA to Treat/Prevent Disease

Biologically active compounds identified using the methods of the invention or a pharmaceutically acceptable salt thereof can be administered to a patient, preferably a mammal, more preferably a human, suffering from a disease whose progression is

associated with a target RNA:host cell factor interaction *in vivo*. In certain embodiments, such compounds or a pharmaceutically acceptable salt thereof is administered to a patient, preferably a mammal, more preferably a human, as a preventative measure against a disease associated with an RNA:host cell factor interaction *in vivo*.

In one embodiment, "treatment" or "treating" refers to an amelioration of a disease, or at least one discernible symptom thereof. In another embodiment, "treatment" or "treating" refers to an amelioration of at least one measurable physical parameter, not necessarily discernible by the patient. In yet another embodiment, "treatment" or "treating" refers to inhibiting the progression of a disease, either physically, *e.g.*, stabilization of a discernible symptom, physiologically, *e.g.*, stabilization of a physical parameter, or both. In yet another embodiment, "treatment" or "treating" refers to delaying the onset of a disease.

In certain embodiments, the compound or a pharmaceutically acceptable salt thereof is administered to a patient, preferably a mammal, more preferably a human, as a preventative measure against a disease associated with an RNA:host cell factor interaction *in vivo*. As used herein, "prevention" or "preventing" refers to a reduction of the risk of acquiring a disease. In one embodiment, the compound or a pharmaceutically acceptable salt thereof is administered as a preventative measure to a patient. According to this embodiment, the patient can have a genetic predisposition to a disease, such as a family history of the disease, or a non-genetic predisposition to the disease. Accordingly, the compound and pharmaceutically acceptable salts thereof can be used for the treatment of one manifestation of a disease and prevention of another.

When administered to a patient, the compound or a pharmaceutically acceptable salt thereof is preferably administered as component of a composition that optionally comprises a pharmaceutically acceptable vehicle. The composition can be administered orally, or by any other convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal, and intestinal mucosa, *etc.*) and may be administered together with another biologically active agent. Administration can be systemic or local. Various delivery systems are known, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, capsules, *etc.*, and can be used to administer the compound and pharmaceutically acceptable salts thereof.

Methods of administration include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, oral, sublingual, intranasal, intracerebral, intravaginal, transdermal, rectally, by inhalation, or topically, particularly to the ears, nose, eyes, or skin. The mode of administration is left to

the discretion of the practitioner. In most instances, administration will result in the release of the compound or a pharmaceutically acceptable salt thereof into the bloodstream.

In specific embodiments, it may be desirable to administer the compound or a pharmaceutically acceptable salt thereof locally. This may be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

In certain embodiments, it may be desirable to introduce the compound or a pharmaceutically acceptable salt thereof into the central nervous system by any suitable route, including intraventricular, intrathecal and epidural injection. Intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent, or via perfusion in a fluorocarbon or synthetic pulmonary surfactant. In certain embodiments, the compound and pharmaceutically acceptable salts thereof can be formulated as a suppository, with traditional binders and vehicles such as triglycerides.

In another embodiment, the compound and pharmaceutically acceptable salts thereof can be delivered in a vesicle, in particular a liposome (see Langer, 1990, Science 249:1527-1533; Treat *et al.*, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*).

In yet another embodiment, the compound and pharmaceutically acceptable salts thereof can be delivered in a controlled release system (see, *e.g.*, Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984)). Other controlled-release systems discussed in the review by Langer, 1990, Science 249:1527-1533) may be used. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald *et al.*, 1980, Surgery 88:507 Saudek *et al.*, 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, 1983, J. Macromol. Sci. Rev. Macromol. Chem. 23:61; see also Levy *et al.*, 1985, Science

228:190; During *et al.*, 1989, Ann. Neurol. 25:351; Howard *et al.*, 1989, J. Neurosurg. 71:105). In yet another embodiment, a controlled-release system can be placed in proximity of a target RNA of the compound or a pharmaceutically acceptable salt thereof, thus requiring only a fraction of the systemic dose.

Compositions comprising the compound or a pharmaceutically acceptable salt thereof ("compound compositions") can additionally comprise a suitable amount of a pharmaceutically acceptable vehicle so as to provide the form for proper administration to the patient.

In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, mammals, and more particularly in humans. The term "vehicle" refers to a diluent, adjuvant, excipient, or carrier with which a compound of the invention is administered. Such pharmaceutical vehicles can be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The pharmaceutical vehicles can be saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea, and the like. In addition, auxiliary, stabilizing, thickening, lubricating and coloring agents may be used. When administered to a patient, the pharmaceutically acceptable vehicles are preferably sterile. Water is a preferred vehicle when the compound of the invention is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid vehicles, particularly for injectable solutions. Suitable pharmaceutical vehicles also include excipients such as starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Compound compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

Compound compositions can take the form of solutions, suspensions, emulsion, tablets, pills, pellets, capsules, capsules containing liquids, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, or any other form suitable for use. In one embodiment, the pharmaceutically acceptable vehicle is a capsule (see *e.g.*, U.S. Patent No. 5,698,155). Other examples of suitable pharmaceutical vehicles are described in Remington's Pharmaceutical Sciences, Alfonso R. Gennaro, ed., Mack Publishing Co. Easton, PA, 19th ed., 1995, pp. 1447 to 1676, incorporated herein by reference.

In a preferred embodiment, the compound or a pharmaceutically acceptable salt thereof is formulated in accordance with routine procedures as a pharmaceutical composition adapted for oral administration to human beings. Compositions for oral delivery may be in the form of tablets, lozenges, aqueous or oily suspensions, granules, powders, emulsions, capsules, syrups, or elixirs, for example. Orally administered compositions may contain one or more agents, for example, sweetening agents such as fructose, aspartame or saccharin; flavoring agents such as peppermint, oil of wintergreen, or cherry; coloring agents; and preserving agents, to provide a pharmaceutically palatable preparation. Moreover, where in tablet or pill form, the compositions can be coated to delay disintegration and absorption in the gastrointestinal tract thereby providing a sustained action over an extended period of time. Selectively permeable membranes surrounding an osmotically active driving compound are also suitable for orally administered compositions. In these later platforms, fluid from the environment surrounding the capsule is imbibed by the driving compound, which swells to displace the agent or agent composition through an aperture. These delivery platforms can provide an essentially zero order delivery profile as opposed to the spiked profiles of immediate release formulations. A time delay material such as glycerol monostearate or glycerol stearate may also be used. Oral compositions can include standard vehicles such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. Such vehicles are preferably of pharmaceutical grade. Typically, compositions for intravenous administration comprise sterile isotonic aqueous buffer. Where necessary, the compositions may also include a solubilizing agent.

In another embodiment, the compound or a pharmaceutically acceptable salt thereof can be formulated for intravenous administration. Compositions for intravenous administration may optionally include a local anesthetic such as lignocaine to lessen pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the compound or a pharmaceutically acceptable salt thereof is to be administered by infusion, it can be dispensed, for example, with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the compound or a pharmaceutically acceptable salt thereof is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The amount of a compound or a pharmaceutically acceptable salt thereof that will be effective in the treatment of a particular disease will depend on the nature of the disease, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed will also depend on the route of administration, and the seriousness of the disease, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for oral administration are generally about 0.001 milligram to about 200 milligrams of a compound or a pharmaceutically acceptable salt thereof per kilogram body weight per day. In specific preferred embodiments of the invention, the oral dose is about 0.01 milligram to about 100 milligrams per kilogram body weight per day, more preferably about 0.1 milligram to about 75 milligrams per kilogram body weight per day, more preferably about 0.5 milligram to 5 milligrams per kilogram body weight per day. The dosage amounts described herein refer to total amounts administered; that is, if more than one compound is administered, or if a compound is administered with a therapeutic agent, then the preferred dosages correspond to the total amount administered. Oral compositions preferably contain about 10% to about 95% active ingredient by weight.

Suitable dosage ranges for intravenous (i.v.) administration are about 0.01 milligram to about 100 milligrams per kilogram body weight per day, about 0.1 milligram to about 35 milligrams per kilogram body weight per day, and about 1 milligram to about 10 milligrams per kilogram body weight per day. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight per day to about 1 mg/kg body weight per day. Suppositories generally contain about 0.01 milligram to about 50 milligrams of a compound of the invention per kilogram body weight per day and comprise active ingredient in the range of about 0.5% to about 10% by weight.

Recommended dosages for intradermal, intramuscular, intraperitoneal, subcutaneous, epidural, sublingual, intracerebral, intravaginal, transdermal administration or administration by inhalation are in the range of about 0.001 milligram to about 200 milligrams per kilogram of body weight per day. Suitable doses for topical administration are in the range of about 0.001 milligram to about 1 milligram, depending on the area of administration. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. Such animal models and systems are well known in the art.

The compound and pharmaceutically acceptable salts thereof are preferably assayed *in vitro* and *in vivo*, for the desired therapeutic or prophylactic activity, prior to use

in humans. For example, *in vitro* assays can be used to determine whether it is preferable to administer the compound, a pharmaceutically acceptable salt thereof, and/or another therapeutic agent. Animal model systems can be used to demonstrate safety and efficacy.

A variety of compounds can be used for treating or preventing diseases in mammals. Types of compounds include, but are not limited to, peptides, peptide analogs including peptides comprising non-natural amino acids, *e.g.*, D-amino acids, phosphorous analogs of amino acids, such as α-amino phosphonic acids and α-amino phosphinic acids, or amino acids having non-peptide linkages, nucleic acids, nucleic acid analogs such as phosphorothioates or peptide nucleic acids ("PNAs"), hormones, antigens, synthetic or naturally occurring drugs, opiates, dopamine, serotonin, catecholamines, thrombin, acetylcholine, prostaglandins, organic molecules, pheromones, adenosine, sucrose, glucose, lactose and galactose.

6. EXAMPLE: THERAPEUTIC TARGETS

The therapeutic targets presented herein are by way of example, and the present invention is not to be limited by the targets described herein. The therapeutic targets presented herein as DNA sequences are understood by one of skill in the art that the sequences can be converted to RNA sequences.

6.1. Tumor Necrosis Factor Alpha ("TNF-α")

GenBank Accession # X01394:

1 gcagaggacc agctaagagg gagagaagca actacagacc ccccctgaaa acaaccctca
61 gacgccacat cccctgacaa gctgccaggc aggttctctt cctctcacat actgacccac
121 ggctccaccc tctctcccct ggaaaggaca ccatgagcac tgaaagcatg atccgggacg
181 tggagctggc cgaggaggcg ctccccaaga agacaggggg gccccagggc tccaggcggt
241 gcttgttcct cagcctcttc tccttcctga tcgtggcagg cgccaccacg ctcttctgcc
301 tgctgcactt tggagtgatc ggcccccaga gggaagagtt ccccagggac ctctctctaa
361 tcagccctct ggcccaggca gtcagatcat cttctcgaac cccgagtgac aagcctgtag
421 cccatgttgt agcaaaccct caagctgagg ggcagctcca gtggctgaac cgccgggcca
481 atgccctcct ggccaatggc gtggagctga gagataacca gctggtggtg ccatcagagg
541 gcctgtacct catctactcc caggtcctct tcaagggcca aggctgcccc tccacccatg
601 tgctcctcac ccacaccatc agccgcatcg ccgtctccta ccagaccaag gtcaacctcc
661 tctctgccat caagagcccc tgccagaggg agaccccaga gggggctgag gccaagccct
721 ggtatgagcc catctatctg ggaggggtct tccagctgga gaagggtgac cgactcagcg
781 ctgagatcaa tcggcccgac tatctcgact ttgccgagtc tgggcaggtc tactttggga

841 tcattgccct gtgaggagga cgaacatcca accttcccaa acgcctcccc tgccccaatc
901 cctttattac cccctccttc agacaccctc aacctcttct ggctcaaaaa gagaattggg
961 ggcttagggt cggaacccaa gcttagaact ttaagcaaca agaccaccac ttcgaaacct
1021 gggattcagg aatgtgtggc ctgcacagtg aattgctggc aaccactaag aattcaaact
1081 ggggcctcca gaactcactg gggcctacag ctttgatccc tgacatctgg aatctggaga
1141 ccagggagcc tttggttctg gccagaatgc tgcaggactt gagaagacct cacctagaaa
1201 ttgacacaag tggaccttag gccttcctct ctccagatgt ttccagactt ccttgagaca
1261 cggagcccag ccctccccat ggagccagct ccctctattt atgtttgcac ttgtgattat
1321 ttattattta tttattattt atttatttac agatgaatgt atttatttgg gagaccgggg
1381 tatcctgggg gacccaatgt aggagctgcc ttggctcaga catgttttcc gtgaaaacgg
1441 agctgaacaa taggctgttc ccatgtagcc ccctggcctc tgtgccttct tttgattatg
1501 tttttaaaa tatttatctg attaagttgt ctaaacaatg ctgatttggt gaccaactgt
1561 cactcattgc tgagcctctg ctccccaggg gagttgtgtc tgtaatcgcc ctactattca
1621 gtggcgagaa ataaagtttg ctt (SEQ ID NO: 6)

General Target Regions:

(1) 5' Untranslated Region - nts 1 - 152
(2) 3' Untranslated Region - nts 852 - 1643

Initial Specific Target Motif:

Group I AU-Rich Element (ARE) Cluster in 3' untranslated region
5' AUUUAUUUAUUUAUUUAUUUA 3' (SEQ ID NO: 1)

6.2. Granulocyte-macrophage Colony Stimulating Factor ("GM-CSF")

GenBank Accession # NM_000758:

1 gctggaggat gtggctgcag agcctgctgc tcttgggcac tgtggcctgc agcatctctg
61 cacccgcccg ctcgcccagc cccagcacgc agccctggga gcatgtgaat gccatccagg
121 aggcccggcg tctcctgaac ctgagtagag acactgctgc tgagatgaat gaaacagtag
181 aagtcatctc agaaatgttt gacctccagg agccgacctg cctacagacc cgcctggagc
241 tgtacaagca gggcctgcgg ggcagcctca ccaagctcaa gggccccttg accatgatgg
301 ccagccacta caagcagcac tgccctccaa ccccggaaac ttcctgtgca acccagacta
361 tcacctttga aagtttcaaa gagaacctga aggactttct gcttgtcatc ccctttgact
421 gctgggagcc agtccaggag tgagaccggc cagatgaggc tggccaagcc ggggagctgc
481 tctctcatga aacaagagct agaaactcag gatggtcatc ttggagggac caaggggtgg
541 gccacagcca tggtgggagt ggcctggacc tgccctgggc cacactgacc ctgatacagg

601 catggcagaa gaatgggaat attttatact gacagaaatc agtaatattt atatatttat
661 atttttaaaa tatttattta tttatttatt taagttcata ttccatattt attcaagatg
721 ttttaccgta ataattatta ttaaaaatat gcttct (SEQ ID NO: 7)

GenBank Accession # XM_003751:

1 tctggaggat gtggctgcag agcctgctgc tcttgggcac tgtggcctgc agcatctctg
61 cacccgcccg ctcgcccagc cccagcacgc agccctggga gcatgtgaat gccatccagg
121 aggcccggcg tctcctgaac ctgagtagag acactgctgc tgagatgaat gaaacagtag
181 aagtcatctc agaaatgttt gacctccagg agccgacctg cctacagacc cgcctggagc
241 tgtacaagca gggcctgcgg ggcagcctca ccaagctcaa gggccccttg accatgatgg
301 ccagccacta caagcagcac tgccctccaa ccccggaaac ttcctgtgca acccagacta
361 tcacctttga aagtttcaaa gagaacctga aggactttct gcttgtcatc ccctttgact
421 gctgggagcc agtccaggag tgagaccggc cagatgaggc tggccaagcc ggggagctgc
481 tctctcatga aacaagagct agaaactcag gatggtcatc ttggagggac caaggggtgg
541 gccacagcca tggtgggagt ggcctggacc tgccctgggc cacactgacc ctgatacagg
601 catggcagaa gaatgggaat attttatact gacagaaatc agtaatattt atatatttat
661 atttttaaaa tatttattta tttatttatt taagttcata ttccatattt attcaagatg
721 ttttaccgta ataattatta ttaaaaatat gcttct (SEQ ID NO: 8)

General Target Regions:

(1) 5' Untranslated Region - nts 1 - 32
(2) 3' Untranslated Region - nts 468 - 789

Initial Specific Target Motif:

Group I AU-Rich Element (ARE) Cluster in 3' untranslated region
5' AUUUAUUUAUUUAUUUAUUUA 3' (SEQ ID NO: 1)

6.3. <u>Interleukin 2 ("IL-2")</u>

GenBank Accession # U25676:

1 atcactctct ttaatcacta ctcacattaa cctcaactcc tgccacaatg tacaggatgc
61 aactcctgtc ttgcattgca ctaattcttg cacttgtcac aaacagtgca cctacttcaa
121 gttcgacaaa gaaaacaaag aaaacacagc tacaactgga gcatttactg ctggatttac
181 agatgatttt gaatggaatt aataattaca agaatcccaa actcaccagg atgctcacat
241 ttaagtttta catgcccaag aaggccacag aactgaaaca gcttcagtgt ctagaagaag
301 aactcaaacc tctggaggaa gtgctgaatt tagctcaaag caaaaacttt cacttaagac

361 ccagggactt aatcagcaat atcaacgtaa tagttctgga actaaaggga tctgaaacaa
421 cattcatgtg tgaatatgca gatgagacag caaccattgt agaatttctg aacagatgga
481 ttaccttttg tcaaagcatc atctcaacac taacttgata attaagtgct tcccacttaa
541 aacatatcag gccttctatt tatttattta aatatttaaa ttttatattt attgttgaat
601 gtatggttgc tacctattgt aactattatt cttaatctta aaactataaa tatggatctt
661 ttatgattct ttttgtaagc cctaggggct ctaaaatggt ttaccttatt tatcccaaaa
721 atatttatta ttatgttgaa tgttaaatat agtatctatg tagattggtt agtaaaacta
781 tttaataaat ttgataaata taaaaaaaaa aaacaaaaaa aaaaa (SEQ ID NO: 9)

General Target Regions:

(1) 5' Untranslated Region - nts 1 - 47
(2) 3' Untranslated Region - nts 519- 825

Initial Specific Target Motifs:

Group III AU-Rich Element (ARE) Cluster in 3' untranslated region
5' NAUUUAUUUAUUUAN 3' (SEQ ID NO: 10)

6.4. Interleukin 6 ("IL-6")

GenBank Accession # NM_000600:

1 ttctgccctc gagcccaccg ggaacgaaag agaagctcta tctcgcctcc aggagcccag
61 ctatgaactc cttctccaca agcgccttcg gtccagttgc cttctccctg gggctgctcc
121 tggtgttgcc tgctgccttc cctgccccag tacccccagg agaagattcc aaagatgtag
181 ccgccccaca cagacagcca ctcacctctt cagaacgaat tgacaaacaa attcggtaca
241 tcctcgacgg catctcagcc ctgagaaagg agacatgtaa caagagtaac atgtgtgaaa
301 gcagcaaaga ggcactggca gaaaacaacc tgaaccttcc aaagatggct gaaaaagatg
361 gatgcttcca atctggattc aatgaggaga cttgcctggt gaaaatcatc actggtcttt
421 tggagtttga ggtataccta gagtacctcc agaacagatt tgagagtagt gaggaacaag
481 ccagagctgt gcagatgagt acaaaagtcc tgatccagtt cctgcagaaa aaggcaaaga
541 atctagatgc aataaccacc cctgacccaa ccacaaatgc cagcctgctg acgaagctgc
601 aggcacagaa ccagtggctg caggacatga caactcatct cattctgcgc agctttaagg
661 agttcctgca gtccagcctg agggctcttc ggcaaatgta gcatgggcac ctcagattgt
721 tgttgttaat gggcattcct tcttctggtc agaaacctgt ccactgggca cagaacttat
781 gttgttctct atggagaact aaaagtatga gcgttaggac actattttaa ttatttttaa
841 tttattaata tttaaatatg tgaagctgag ttaatttatg taagtcatat ttatattttt
901 aagaagtacc acttgaaaca ttttatgtat tagttttgaa ataataatgg aaagtggcta

961 tgcagtttga atatcctttg tttcagagcc agatcatttc ttggaaagtg taggcttacc
1021 tcaaataaat ggctaactta tacatatttt taaagaaata tttatattgt atttatataa
1081 tgtataaatg gtttttatac caataaatgg cattttaaaa aattc (SEQ ID NO: 11)

General Target Regions:

(1) 5' Untranslated Region - nts 1 - 62

(2) 3' Untranslated Region - nts 699 - 1125

Initial Specific Target Motifs:

Group III AU-Rich Element (ARE) Cluster in 3' untranslated region
5' NAUUUAUUUAUUUAN 3' (SEQ ID NO: 10)

6.5. Vascular Endothelial Growth Factor ("VEGF")

GenBank Accession # AF022375:

1 aagagctcca gagagaagtc gaggaagaga gagacggggt cagagagagc gcgcgggcgt
61 gcgagcagcg aaagcgacag gggcaaagtg agtgacctgc ttttgggggt gaccgccgga
121 gcgcggcgtg agccctcccc cttgggatcc cgcagctgac cagtcgcgct gacggacaga
181 cagacagaca ccgcccccag ccccagttac cacctcctcc ccggccggcg gcggacagtg
241 gacgcggcgg cgagccgcgg gcaggggccg gagcccgccc ccggaggcgg ggtggagggg
301 gtcggagctc gcggcgtcgc actgaaactt ttcgtccaac ttctgggctg ttctcgcttc
361 ggaggagccg tggtccgcgc gggggaagcc gagccgagcg gagccgcgag aagtgctagc
421 tcgggccggg aggagccgca gccggaggag ggggaggagg aagaagagaa ggaagaggag
481 agggggccgc agtggcgact cggcgctcgg aagccgggct catggacggg tgaggcggcg
541 gtgtgcgcag acagtgctcc agcgcgcgcg ctccccagcc ctggcccggc ctcgggccgg
601 gaggaagagt agctcgccga ggcgccgagg agagcgggcc gccccacagc ccgagccgga
661 gagggacgcg agccgcgcgc cccggtcggg cctccgaaac catgaacttt ctgctgtctt
721 gggtgcattg gagccttgcc ttgctgctct acctccacca tgccaagtgg tcccaggctg
781 cacccatggc agaaggagga gggcagaatc atcacgaagt ggtgaagttc atggatgtct
841 atcagcgcag ctactgccat ccaatcgaga ccctggtgga catcttccag gagtaccctg
901 atgagatcga gtacatcttc aagccatcct gtgtgcccct gatgcgatgc gggggctgct
961 ccaatgacga gggcctggag tgtgtgccca ctgaggagtc caacatcacc atgcagatta
1021 tgcggatcaa acctcaccaa ggccagcaca taggagagat gagcttccta cagcacaaca
1081 aatgtgaatg cagaccaaag aaagatagag caagacaaga aaatccctgt gggccttgct
1141 cagagcggag aaagcatttg tttgtacaag atccgcagac gtgtaaatgt tcctgcaaaa
1201 acacacactc gcgttgcaag gcgaggcagc ttgagttaaa cgaacgtact tgcagatgtg

1261 acaagccgag gcggtgagcc gggcaggagg aaggagcctc cctcagggtt tcgggaacca
1321 gatctctctc caggaaagac tgatacagaa cgatcgatac agaaaccacg ctgccgccac
1381 cacaccatca ccatcgacag aacagtcctt aatccagaaa cctgaaatga aggaagagga
1441 gactctgcgc agagcacttt gggtccggag ggcgagactc cggcggaagc attcccgggc
1501 gggtgaccca gcacggtccc tcttggaatt ggattcgcca ttttattttt cttgctgcta
1561 aatcaccgag cccggaagat tagagagttt tatttctggg attcctgtag acacacccac
1621 ccacatacat acatttatat atatatatat tatatatata taaaaataaa tatctctatt
1681 ttatatatat aaaatatata tattcttttt ttaaattaac agtgctaatg ttattggtgt
1741 cttcactgga tgtatttgac tgctgtggac ttgagttggg aggggaatgt tcccactcag
1801 atcctgacag ggaagaggag gagatgagag actctggcat gatctttttt ttgtcccact
1861 tggtggggcc agggtcctct cccctgccca agaatgtgca aggccagggc atgggggcaa
1921 atatgaccca gttttgggaa caccgacaaa cccagccctg gcgctgagcc tctctacccc
1981 aggtcagacg gacagaaaga caaatcacag gttccgggat gaggacaccg gctctgacca
2041 ggagtttggg gagcttcagg acattgctgt gctttgggga ttccctccac atgctgcacg
2101 cgcatctcgc ccccaggggc actgcctgga agattcagga gcctgggcgg ccttcgctta
2161 ctctcacctg cttctgagtt gcccaggagg ccactggcag atgtcccggc gaagagaaga
2221 gacacattgt tggaagaagc agcccatgac agcgcccctt cctgggactc gccctcatcc
2281 tcttcctgct ccccttcctg ggtgcagcc taaaaggacc tatgtcctca caccattgaa
2341 accactagtt ctgtcccccc aggaaacctg gttgtgtgtg tgtgagtggt tgaccttcct
2401 ccatcccctg gtccttccct tcccttcccg aggcacagag agacagggca ggatccacgt
2461 gcccattgtg gaggcagaga aaagagaaag tgttttatat acggtactta tttaatatcc
2521 ctttttaatt agaaattaga acagttaatt taattaaaga gtagggtttt ttttcagtat
2581 tcttggttaa tatttaattt caactattta tgagatgtat cttttgctct ctcttgctct
2641 cttatttgta ccggtttttg tatataaaat tcatgtttcc aatctctctc tccctgatcg
2701 gtgacagtca ctagcttatc ttgaacagat atttaatttt gctaacactc agctctgccc
2761 tccccgatcc cctggctccc cagcacacat tcctttgaaa gagggtttca atatacatct
2821 acatactata tatatattgg gcaacttgta tttgtgtgta tatatatata tatatgttta
2881 tgtatatatg tgatcctgaa aaaataaaca tcgctattct gttttttata tgttcaaacc
2941 aaacaagaaa aaatagagaa ttctacatac taaatctctc tccttttta attttaatat
3001 ttgttatcat ttatttattg gtgctactgt ttatccgtaa taattgtggg gaaaagatat
3061 taacatcacg tctttgtctc tagtgcagtt tttcgagata ttccgtagta catatttatt
3121 tttaaacaac gacaaagaaa tacagatata tcttaaaaaa aaaaaa (SEQ ID NO: 12)

General Target Regions:

(1) 5' Untranslated Region - nts 1 - 701

(2) 3' Untranslated Region - nts 1275 - 3166

Initial Specific Target Motifs:

(1) Internal Ribosome Entry Site (IRES) in 5' untranslated region nts 513 -704
5'CCGGGCUCAUGGACGGGUGAGGCGGCGGUGUGCGCAGACAGU GCUCCAGCGCGCGCGCUCCCCAGCCCUGGCCCGGCCUCGGGCCG GGAGGAAGAGUAGCUCGCCGAGGCGCCGAGGAGAGCGGGCCGC CCCACAGCCCGAGCCGGAGAGGGACGCGAGCCGCGCGCCCCGGU CGGGCCUCCGAAACCAUGAACUUUCUGCUGUCUUGGGUGCAUU GGAGCCUUGCCUUGCUGCUCUACCUCCACCAUG 3' (SEQ ID NO: 13)

(2) Group III AU-Rich Element (ARE) Cluster in 3' untranslated region
5' NAUUUAUUUAUUUAN 3' (SEQ ID NO: 10)

6.6. Human Immunodeficiency Virus I ("HIV-1")

GenBank Accession # NC_001802:

1 ggtctctctg gttagaccag atctgagcct gggagctctc tggctaacta gggaacccac
61 tgcttaagcc tcaataaagc ttgccttgag tgcttcaagt agtgtgtgcc cgtctgttgt
121 gtgactctgg taactagaga tccctcagac ccttttagtc agtgtggaaa atctctagca
181 gtggcgcccg aacagggacc tgaaagcgaa agggaaacca gaggagctct ctcgacgcag
241 gactcggctt gctgaagcgc gcacggcaag aggcgagggg cggcgactgg tgagtacgcc
301 aaaaattttg actagcggag gctagaagga gagagatggg tgcgagagcg tcagtattaa
361 gcgggggaga attagatcga tgggaaaaaa ttcggttaag gccaggggga aagaaaaaat
421 ataaattaaa acatatagta tgggcaagca gggagctaga acgattcgca gttaatcctg
481 gcctgttaga aacatcagaa ggctgtagac aaatactggg acagctacaa ccatcccttc
541 agacaggatc agaagaactt agatcattat ataatacagt agcaaccctc tattgtgtgc
601 atcaaaggat agagataaaa gacaccaagg aagctttaga caagatagag gaagagcaaa
661 acaaaagtaa gaaaaaagca cagcaagcag cagctgacac aggacacagc aatcaggtca
721 gccaaaatta ccctatagtg cagaacatcc aggggcaaat ggtacatcag gccatatcac
781 ctagaacttt aaatgcatgg gtaaaagtag tagaagagaa ggctttcagc ccagaagtga
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9121 ctctctggct aactagggaa cccactgctt aagcctcaat aaagcttgcc ttgagtgctt
9181 c (SEQ ID NO: 14)

Initial Specific Target Motifs:

(1) Trans-activation response region/Tat protein binding site - TAR RNA - nts 1 - 60

"Minimal" TAR RNA element

5' GGCAGAUCUGAGCCUGGGAGCUCUCUGCC 3' (SEQ ID NO: 15)

(2) Gag/Pol Frameshifting Site - "Minimal" frameshifting element
5'
UUUUUUAGGGAAGAUCUGGCCUUCCUACAAGGGAAGGCCAGG
GAAUUUUCUU 3' (SEQ ID NO: 16)

6.7. Hepatitis C Virus ("HCV" - Genotypes 1a & 1b)

GenBank Accession # NC_001433:

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5041 acttcttgtc ccagaccaaa caggcaggag acaacctccc ctacctggta gcataccaag
5101 ccacagtgtg cgccagggct caggctccac ctccatcgtg ggaccaaatg tggaagtgtc
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5221 ttcaaaatga ggtcactctc acacacccca taaccaaata catcatggca tgcatgtcgg
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5341 ccgcgtactg cctgacgaca ggcagcgtgg tcattgtggg caggatcatc ttgtccggga
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5701 tggcttttac agcctctatc accagcccgc tcaccaccca aaataccctc ctgtttaaca
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7441 aggcctccga cgacggcgac aaaggatccg acgttgagtc gtactcctcc atgcccccc
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7621 cgccatgcgc tgcggaggag agcaagttgc ccatcaatcc gttgagcaac tctttgctgc
7681 gtcaccacag tatggtctac tccacaacat ctcgcagcgc aagtctgcgg cagaagaagg
7741 tcacctttga cagactgcaa gtcctggacg accactaccg ggacgtgctc aaggagatga
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7861 cgccccccaca ttcggccaaa tccaaatttg gctacggggc gaaggacgtc cggagcctat

7921 ccagcagggc cgtcaaccac atccgctccg tgtgggagga cttgctggaa gacactgaaa
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8281 acatccgtac tgaggaatca atttaccaat gttgtgactt ggcccccgaa gccaggcagg
8341 ccataaggtc gctcacagag cggctttatg tcgggggtcc cctgactaat tcgaaggggc
8401 agaactgcgg ttatcgccgg tgccgcgcaa gtggcgtgct gacgactagc tgcggcaaca
8461 ccctcacatg ttacttgaag gccactgcgg cctgtcgagc tgcaaagctc caggactgca
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8641 acccgcccca accagaatac gacttggagc tgataacgtc atgctcctcc aatgtgtcgg
8701 tcgcgcacga tgcatccggc aaaagggtgt actacctcac ccgtgacccc accacccccc
8761 tcgcacgggc tgcgtgggag acagttagac acactccagt caactcctgg ctaggcaata
8821 tcatcatgta tgcgcccacc ctatgggcga ggatgattct gatgactcat ttcttctcta
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8941 actccattga gccacttgac ctacctcaga tcattgaacg actccatggt cttagcgcat
9001 tttcactcca cagttactct ccaggtgaga tcaatagggt ggcttcatgc ctcaggaaac
9061 ttggggtacc gcctttgcga gtctggagac atcgggccag aagtgtccgc gctaagctac
9121 tgtcccaggg ggggagggct gccacttgcg gcaagtacct cttcaactgg gcagtaaaga
9181 ccaagcttaa actcactcca atcccggctg cgtcccagct agacttgtcc ggctggttcg
9241 ttgctggtta caacggggga gacatatatc acagcctgtc tcgtgcccga ccccgttggt
9301 tcatgttgtg cctactccta ctttctgtag ggtaggcat ctacctgctc cccaaccggt
9361 gaacggggag ctaaccactc caggccaata ggccattccc ttttttttt ttc (SEQ ID NO: 17)

General Target Region:

5' Untranslated Region - nts 1 - 328 - Internal Ribosome Entry Site (IRES):

5'UUGGGGGCGACACUCCACCAUAGAUCACUCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAGCGUCUAGCCAUGGCGUUAGUAUGAGUGUUGUGCAGCCUCCAGGACCCCCCCUCCCGGGAGAGCCAUAGUGGUCUGCGGAACCGGUGAGUACACCGGAAUUGCCAGGACGACCGGGUCCUUUCUUGGAUCAACCCGCUCAAUGCCUGGAGAUUUGGGCGUGCCCCCGCGAGACUGCUAGCCGAGUAGUGUUGGGUCGCGAAAGGCCUUGUGGUACUGCCUGAUAGGGUGCUUGCGAGUGCCCCGGGAGGUCUCGUAGACCGUGCAU3' (SEQ ID NO: 18)

Initial Specific Target Motifs:

(1) Subdomain IIIc within HCV IRES - nts 213 - 226
5'AUUUGGGCGUGCCC3' (SEQ ID NO: 19)

(2) Subdomain IIId within HCV IRES - nts 241-267
5'GCCGAGUAGUGUUGGGUCGCGAAAGGC3' (SEQ ID NO: 20)

6.8. Ribonuclease P RNA ("RNaseP")

GenBank Accession #s

X15624 Homo sapiens RNaseP H1 RNA:

1 atgggcggag ggaagctcat cagtggggcc acgagctgag tgcgtcctgt cactccactc
61 ccatgtccct tgggaaggtc tgagactagg gccagaggcg gccctaacag ggctctccct
121 gagcttcagg gaggtgagtt cccagagaac ggggctccgc gcgaggtcag actgggcagg
181 agatgccgtg gaccccgccc ttcggggagg ggcccggcgg atgcctcctt tgccggagct
241 tggaacagac tcacggccag cgaagtgagt tcaatggctg aggtgaggta ccccgcaggg
301 gacctcataa cccaattcag accactctcc tccgccccatt (SEQ ID NO: 21)

U64885 Staphylococcus aureus RNaseP (rrnB) RNA:

1 gaggaaagtc cgggctcaca cagtctgaga tgattgtagt gttcgtgctt gatgaaacaa
61 taaatcaagg cattaatttg acggcaatga aatatcctaa gtctttcgat atggatagag
121 taatttgaaa gtgccacagt gacgtagctt ttatagaaat ataaaaggtg gaacgcggta
181 aacccctcga gtgagcaatc caaatttggt aggagcactt gtttaacgga attcaacgta
241 taaacgagac acacttcgcg aaatgaagtg gtgtagacag atggttatca cctgagtacc
301 agtgtgacta gtgcacgtga tgagtacgat ggaacagaac gcggcttat (SEQ ID NO: 22)

M17569 Escherichia coli RNA component (M1 RNA) of ribonuclease P (rnpB) gene:

1 gaagctgacc agacagtcgc cgcttcgtcg tcgtcctctt cgggggagac gggcggaggg
61 gaggaaagtc cgggctccat agggcagggt gccaggtaac gcctgggggg gaaacccacg
121 accagtgcaa cagagagcaa accgccgatg gcccgcgcaa gcgggatcag gtaagggtga
181 aagggtgcgg taagagcgca ccgcgcggct ggtaacagtc cgtggcacgg taaactccac
241 ccggagcaag gccaaatagg ggttcataag gtacggcccg tactgaaccc gggtaggctg
301 cttgagccag tgagcgattg ctggcctaga tgaatgactg tccacgacag aacccggctt
361 atcggtcagt ttcacct (SEQ ID NO: 23)

Z70692 Mycobacterium tuberculosis RNaseP (rnpB) RNA:

1 ccaccggtta cgatcttgcc gaccatggcc ccacaatagg gccggggaga cccggcgtca
61 gtggtgggcg gcacggtcag taacgtctgc gcaacacggg gttgactgac gggcaatatc
121 ggctccatag cgtcggccgc ggatacagta aaggagcatt ctgtgacgga aaagacgccc
181 gacgacgtct tcaaacttgc caaggacgag aaggtcgaat atgtcgacgt ccggttctgt
241 gacctgcctg gcatcatgca gcacttcacg attccggctt cggcctttga caagagcgtg
301 tttgacgacg gcttggcctt tgacggctcg tcgattcgcg ggttccagtc gatccacgaa
361 tccgacatgt tgcttcttcc cgatcccgag acggcgcgca tcgacccgtt ccgcgcggcc
421 aagacgctga atatcaactt ctttgtgcac gacccgttca ccctggagcc gtactcccgc
481 gacccgcgca acatcgcccg caaggccgag aactacctga tcagcactgg catcgccgac
541 accgcatact tcggcgccga ggccgagttc tacattttcg attcggtgag cttcgactcg
601 cgcgccaacg gctccttcta cgaggtggac gccatctcgg ggtggtggaa caccggcgcg
661 gcgaccgagg ccgacggcag tcccaaccgg ggctacaagg tccgccacaa gggcgggtat
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1021 ccgctgatgt acgacgagac gggttatgcc ggtctgtcgg acacggcccg tcattacatc
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1141 tacaagcggc tggttcccgg ttacgaggcc ccgatcaacc tggtctatag ccagcgcaac
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1921 ctcccgaaca ctttgcgcac taccgccttt gcccgccgcg tcacccgtag gtagttgtcc
1981 aggaattccc caccgtcgtc gtttcgccag ccggccgcga ccgcgaccgc attgagctgg

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33181 cgggcaatta ccggtatgcg atcaataact cgttcggatt cggcggccac aacgtggcaa
33241 tcgccttcgg acggtactaa accccagcgt tacgcgacag gagacctgcg atgacaatca
33301 tggcccccga ggcggttggc gagtcgctcg acccccgcga tccgctgttg cggctgagca
33361 acttcttcga cgacggcagc gtggaattgc tgcacgagcg tgaccgctcc ggagtgctgg
33421 ccgcggcggg caccgtcaac ggtgtgcgca ccatcgcgtt ctgcaccgac ggcaccgtga
33481 tgggcggcgc catgggcgtc gaggggtgca cgcacatcgt caacgcctac gacactgcca

33541 tcgaagacca gagtcccatc gtgggcatct ggcattcggg tggtgcccgg ctggctgaag
33601 gtgtgcgggc gctgcacgcg gtaggccagg tgttcgaagc catgatccgc gcgtccggct
33661 acatcccgca gatctcggtg gtcgtcggtt tcgccgccgg cggcgccgcc tacggaccgg
33721 cgttgaccga cgtcgtcgtc atggcgccgg aaagccgggt gttcgtcacc gggcccgacg
33781 tggtgcgcag cgtcaccggc gaggacgtcg acatggcctc gctcggtggg ccggagaccc
33841 accacaagaa gtccggggtg tgccacatcg tcgccgacga cgaactcgat gcctacgacc
33901 gtgggcgccg gttggtcgga ttgttctgcc agcaggggca tttcgatcgc agcaaggccg
33961 aggccggtga caccgacatc cacgcgctgc tgccggaatc ctcgcgacgt gcctacgacg
34021 tgcgtccgat cgtgacggcg atcctcgatg cggacacacc gttcgacgag ttccaggcca
34081 attgggcgcc gtcgatggtg gtcgggctgg gtcggctgtc gggtcgcacg gtgggtgtac
34141 tggccaacaa cccgctacgc ctgggcggct gcctgaactc cgaaagcgca gagaaggcag
34201 cgcgtttcgt gcggctgtgc gacgcgttcg ggattccgct ggtggtggtg gtcgatgtgc
34261 cgggctatct gcccggtgtc gaccaggagt ggggtggcgt ggtgcgccgt ggcgccaagt
34321 tgctgcacgc gttcggcgag tgcaccgttc cgcgggtcac gctggtcacc cgaaagacct
34381 acggcggggc atacattgcg atgaactccc ggtcgttgaa cgcgaccaag gtgttcgcct
34441 ggccggacgc cgaggtcgcg gtgatgggcg ctaaggcggc cgtcggcatc ctgcacaaga
34501 agaagttggc cgccgctccg gagcacgaac gcgaagcgct gcacgaccag ttggccgccg
34561 agcatgagcg catcgccggc ggggtcgaca gtgcgctgga catcggtgtg gtcgacgaga
34621 agatcgaccc ggcgcatact cgcagcaagc tcaccgaggc gctggcgcag gctccggcac
34681 ggcgcggccg ccacaagaac atcccgctgt agttctgacc gcgagcagac gcagaatcgc
34741 acgcgcgagg tccgcgccgt gcgattctgc gtctgctcgc cagttatccc cagcggtggc
34801 tggtcaacgc gaggcgctcc tcgcatgctc ggacggtgcc taccgacgcg ctaacaattc
34861 tcgagaaggc cggcgggttc gccaccaccg cgcaattgct cacggtcatg acccgccaac
34921 agctcgacgt ccaagtgaaa aacggcggcc tcgttcgcgt ttggtacggg gtctacgcgg
34981 cacaagagcc ggacctgttg ggccgcttgg cggctctcga tgtgttcatg ggggggcacg
35041 ccgtcgcgtg tctgggcacc gccgccgcgt tgtatggatt cgacacggaa aacaccgtcg
35101 ctatccatat gctcgatccc ggagtaagga tgcggcccac ggtcggtctg atggtccacc
35161 aacgcgtcgg tgcccggctc caacgggtgt caggtcgtct cgcgaccgcg cccgcatgga
35221 ctgccgtgga ggtcgcacga cagttgcgcc gcccgcgggc gctggccacc ctcgacgccg
35281 cactacggtc aatgcgctgc gctcgcagtg aaattgaaaa cgccgttgct gagcagcgag
35341 gccgccgagg catcgtcgcg gcgcgcgaac tcttaccctt cgccgacgga cgcgcggaat
35401 cggccatgga gagcgaggct cggctcgtca tgatcgacca cgggctgccg ttgcccgaac
35461 ttcaataccc gatacacggc cacggtggtg aaatgtggcg agtcgacttc gcctggcccg
35521 acatgcgtct cgcggccgaa tacgaaagca tcgagtggca cgcgggaccg gcggagatgc
35581 tgcgcgacaa gacacgctgg gccaagctcc aagagctcgg gtggacgatt gtcccgattg

35641 tcgtcgacga tgtcagacgc gaacccggcc gcctggcggc ccgcatcgcc cgccacctcg
35701 accgcgcgcg tatggccggc tgaccgctgg tgagcagacg cagagtcgca ctgcggccgg
35761 cgcagtgcga ctctgcgtct gctcgcgctc aacggctgag gaactcctta gccacggcga
35821 ctacgcgctc gcgatcccgt ggcaccagac cgatccgggt ccggcggtcg aggatatcgt
35881 ccacatccag cgccccctca tgggtcaccg cgtattcgaa ctccgcccgg gtcacgtcga
35941 tgccgtcggc gaccggctcg gtgggccgct cacatgtggc ggcggcagcg acgttggccg
36001 cctcggcccc gtaccgcgcc accagcgact cgggcaatcc ggcgcccgat ccgggggccg
36061 gcccagggtt cgccggtgcg ccgatcagcg gcaggttgcg agtgcggcac ttcgcggctc
36121 gcaggtgtcg cagcgtgatg gcgcgattca gcacatcctc tgccatgtag cggtattccg
36181 tcagcttgcc gccgaccaca ctgatcacgc ccgacggcga ttcaaaaaca gcgtggtcac
36241 gcgaaacgtc ggcggtgcgg ccctggacac cagcaccgcc ggtgtcgatt agcggccgca
36301 atcccgcata ggcaccgatg acatccttgg tgccgaccgc cgtccccaat gcggtgttca
36361 ccgtatccag caggaacgtg atctcttccg aagacggttg tggcacatcg ggaatcgggc
36421 cgggtgcgtc ttcgtcggtc agcccgagat agatccggcc cagctgctcg ggcatggcga
36481 acacgaagcg gttcagctca ccggggatcg gaatggtcag cgcggcagtc ggattggcaa
36541 acgacttcgc gtcgaagacc agatgtgtgc cgcggctggg gcgtagcctc agggacgggt
36601 cgatctcacc cgcccacacg cccgccgcgt tgatgacggc acgcgccgac agcgcgaacg
36661 actgccgggt gcgccggtcg gtcaactcca ccgaagtgcc ggtgacattc gacgcgccca
36721 cgtaagtgag gatgcgggcg ccgtgctggg ccgcggtgcg cgcgacggcc atgaccagcc
36781 gggcgtcgtc gatcaattgc ccgtcgtacg cgagcagacc accgtcgagg ccgtcccgcc
36841 gaacggtggg agcaatctcc accacccgtg acgccgggat tcggcgcgat cggggcaacg
36901 tcgccgccgg cgtacccgct agcacccgca aagcgtcgcc ggccaggaaa ccggcacgca
36961 ccaacgcccg cttggtgtga cccatcgacg gcaacaacgg gaccagttgc ggcatggcat
37021 gcacgagatg aggagcgttg cgtgtcatca ggattccgcg ttcgacggcg ctgcgccggg
37081 cgatgcccac gttgccgctg gccagatagc gcagaccgcc gtgcaccaac ttcgagctcc
37141 agcggctggt gccgaacgcc agatcatgct tttccaccaa ggccaccgtc agaccgcggg
37201 tggcagcatc taaggcaatg ccaacaccgg taatgccgcc gcctatcacg atgacgtcga
37261 gtgcgccacc gtcggccagt gcggtcaggt cggcggagcg acgcgccgcg ttgagtgcag
37321 ccgagtgggg catcagcaca aatatccgtt cagtgcgtgg gtaagttcgg tggccagcgc
37381 ggcggaatcg aggatcgaat cgacgatgtc cgcggactgg atggtcgact gggcgatcag
37441 caacaccatg gtcgccagtc gacgagcgtc gccggagcgc acactgcccg accgctgcgc
37501 cactgtcagc cgggcggcca acccctcgat caggacctgc tggctggtgc cgaggcgctc
37561 ggtgatgtac accctggcca gctccgagtg catgaccgac atgatcagat cgtcaccccg
37621 caaccggtcg gccaccgcga caatctgctt taccaacgct tcccggtcgt ccccgtcgag
37681 gggcacctcc cgcagcacgt cggcgatatg gctggtcagc atggacgcca tgatcgaccg

37741 ggtgtccggc cagcgacggt atacggtcgg gcggctcacg cccgcgcgcc gggcgatctc
37801 ggcaagtgtc acccggtcca cgccgtaatc gacgacgcag ctcgccgctg cccgcaggat
37861 acgaccaccg gtatccgcgc ggtcattact cattgacagc atgtgtaata ctgtaacgcg
37921 tgactcaccg cgaggaactc cttccaccga tgaaatggga cgcgtgggga gatcccgccg
37981 cggccaagcc actttctgat ggcgtccggt cgttgctgaa gcaggttgtg ggcctagcgg
38041 actcggagca gcccgaactc gaccccgcgc aggtgcagct gcgcccgtcc gccctgtcgg
38101 gggcagacca (SEQ ID NO: 24)

6.9. X-linked Inhibitor of Apoptosis Protein ("XIAP")

GenBank Accession # U45880:

1 gaaaaggtgg acaagtccta ttttcaagag aagatgactt ttaacagttt tgaaggatct
61 aaaacttgtg tacctgcaga catcaataag gaagaagaat ttgtagaaga gtttaataga
121 ttaaaaactt ttgctaattt tccaagtggt agtcctgttt cagcatcaac actggcacga
181 gcagggtttc tttatactgg tgaaggagat accgtgcggt gctttagttg tcatgcagct
241 gtagatagat ggcaatatgg agactcagca gttggaagac acaggaaagt atccccaaat
301 tgcagattta tcaacggctt ttatcttgaa aatagtgcca cgcagtctac aaattctggt
361 atccagaatg gtcagtacaa agttgaaaac tatctgggaa gcagagatca ttttgcctta
421 gacaggccat ctgagacaca tgcagactat cttttgagaa ctgggcaggt tgtagatata
481 tcagacacca tatacccgag gaaccctgcc atgtattgtg aagaagctag attaaagtcc
541 tttcagaact ggccagacta tgctcaccta accccaagag agttagcaag tgctggactc
601 tactacacag gtattggtga ccaagtgcag tgcttttgtt gtggtggaaa actgaaaaat
661 tgggaacctt gtgatcgtgc ctggtcagaa cacaggcgac actttcctaa ttgcttcttt
721 gttttgggcc ggaatcttaa tattcgaagt gaatctgatg ctgtgagttc tgataggaat
781 ttcccaaatt caacaaatct tccaagaaat ccatccatgg cagattatga agcacggatc
841 tttacttttg ggacatggat atactcagtt aacaaggagc agcttgcaag agctggattt
901 tatgctttag gtgaaggtga taaagtaaag tgctttcact gtggaggagg gctaactgat
961 tggaagccca gtgaagaccc ttgggaacaa catgctaaat ggtatccagg gtgcaaatat
1021 ctgttagaac agaagggaca agaatatata aacaatattc atttaactca ttcacttgag
1081 gagtgtctgg taagaactac tgagaaaaca ccatcactaa ctagaagaat tgatgatacc
1141 atcttccaaa atcctatggt acaagaagct atacgaatgg ggttcagttt caaggacatt
1201 aagaaaataa tggaggaaaa aattcagata tctgggagca actataaatc acttgaggtt
1261 ctggttgcag atctagtgaa tgctcagaaa gacagtatgc aagatgagtc aagtcagact
1321 tcattacaga aagagattag tactgaagag cagctaaggc gcctgcaaga ggagaagctt
1381 tgcaaaatct gtatggatag aaatattgct atcgtttttg ttccttgtgg acatctagtc
1441 acttgtaaac aatgtgctga agcagttgac aagtgtccca tgtgctacac agtcattact

1501 ttcaagcaaa aaatttttat gtcttaatct aactctatag taggcatgtt atgttgttct
1561 tattaccctg attgaatgtg tgatgtgaac tgactttaag taatcaggat tgaattccat
1621 tagcatttgc taccaagtag gaaaaaaaat gtacatggca gtgttttagt tggcaatata
1681 atctttgaat ttcttgattt ttcagggtat tagctgtatt atccattttt tttactgtta
1741 tttaattgaa accatagact aagaataaga agcatcatac tataactgaa cacaatgtgt
1801 attcatagta tactgattta atttctaagt gtaagtgaat taatcatctg gattttttat
1861 tcttttcaga taggcttaac aaatggagct ttctgtatat aaatgtggag attagagtta
1921 atctccccaa tcacataatt tgttttgtgt gaaaaaggaa taaattgttc catgctggtg
1981 gaaagataga gattgttttt agaggttggt tgttgtgttt taggattctg tccatttttct
2041 tgtaaaggga taaacacgga cgtgtgcgaa atatgtttgt aaagtgattt gccattgttg
2101 aaagcgtatt taatgataga atactatcga gccaacatgt actgacatgg aaagatgtca
2161 gagatatgtt aagtgtaaaa tgcaagtggc gggacactat gtatagtctg agccagatca
2221 aagtatgtat gttgttaata tgcatagaac gagagatttg gaaagatata caccaaactg
2281 ttaaatgtgg tttctcttcg gggagggggg gattggggga ggggccccag aggggtttta
2341 gaggggcctt ttcactttcg actttttca ttttgttctg ttcggatttt ttataagtat
2401 gtagaccccg aagggtttta tgggaactaa catcagtaac ctaaccccccg tgactatcct
2461 gtgctcttcc tagggagctg tgttgtttcc cacccaccac ccttccctct gaacaaatgc
2521 ctgagtgctg gggcactttg (SEQ ID NO: 25)

General Target Region:

Internal Ribosome Entry Site (IRES) in 5' untranslated region:
5'AGCUCCUAUAACAAAAGUCUGUUGCUUGUGUUUCACAUUUUGGAUU UCCUAAUAUAAUGUUCUCUUUUUAGAAAAGGUGGACAAGUCCUAUUU UCAAGAGAAG3' (SEQ ID NO: 26)

Initial Specific Target Motif:

RNP core binding site within XIAP IRES
5'GGAUUUCCUAAUAUAAUGUUCUCUUUUU3' (SEQ ID NO: 27)

6.10. Survivin

GenBank Accession # NM_001168:

1 ccgccagatt tgaatcgcgg gacccgttgg cagaggtggc ggcggcggca tgggtgcccc
61 gacgttgccc cctgcctggc agccctttct caaggaccac cgcatctcta cattcaagaa
121 ctggcccttc ttggagggct gcgcctgcac cccggagcgg atggccgagg ctggcttcat
181 ccactgcccc actgagaacg agccagactt ggcccagtgt ttcttctgct tcaaggagct

241 ggaaggctgg gagccagatg acgaccccat agaggaacat aaaaagcatt cgtccggttg
301 cgctttcctt tctgtcaaga agcagtttga agaattaacc cttggtgaat ttttgaaact
361 ggacagagaa agagccaaga acaaaattgc aaaggaaacc aacaataaga agaaagaatt
421 tgaggaaact gcgaagaaag tgcgccgtgc catcgagcag ctggctgcca tggattgagg
481 cctctggccg gagctgcctg gtcccagagt ggctgcacca cttccagggt ttattccctg
541 gtgccaccag ccttcctgtg ggccccttag caatgtctta ggaaaggaga tcaacatttt
601 caaattagat gtttcaactg tgctcctgtt ttgtcttgaa agtggcacca gaggtgcttc
661 tgcctgtgca gcgggtgctg ctggtaacag tggctgcttc tctctctctc tctctttttt
721 gggggctcat ttttgctgtt ttgattcccg ggcttaccag gtgagaagtg agggaggaag
781 aaggcagtgt cccttttgct agagctgaca gctttgttcg cgtgggcaga gccttccaca
841 gtgaatgtgt ctggacctca tgttgttgag gctgtcacag tcctgagtgt ggacttggca
901 ggtgcctgtt gaatctgagc tgcaggttcc ttatctgtca cacctgtgcc tcctcagagg
961 acagtttttt tgttgttgtg tttttttgtt tttttttttt ggtagatgca tgacttgtgt
1021 gtgatgagag aatggagaca gagtccctgg ctcctctact gtttaacaac atggctttct
1081 tattttgttt gaattgttaa ttcacagaat agcacaaact acaattaaaa ctaagcacaa
1141 agccattcta agtcattggg gaaacggggt gaacttcagg tggatgagga gacagaatag
1201 agtgatagga agcgtctggc agatactcct tttgccactg ctgtgtgatt agacaggccc
1261 agtgagccgc ggggcacatg ctggccgctc ctccctcaga aaaaggcagt ggcctaaatc
1321 ctttttaaat gacttggctc gatgctgtgg gggactggct gggctgctgc aggccgtgtg
1381 tctgtcagcc caaccttcac atctgtcacg ttctccacac gggggagaga cgcagtccgc
1441 ccaggtcccc gctttctttg gaggcagcag ctcccgcagg gctgaagtct ggcgtaagat
1501 gatggatttg attcgccctc ctccctgtca tagagctgca gggtggattg ttacagcttc
1561 gctggaaacc tctggaggtc atctcggctg ttcctgagaa ataaaaagcc tgtcatttc (SEQ ID NO: 28)

7. EXAMPLE: IDENTIFICATION OF A DYE-LABELED TARGET RNA BOUND TO SMALL MOLECULAR WEIGHT COMPOUNDS

The results presented in this Example indicate that gel mobility shift assays can be used to detect the binding of small molecules, such as the Tat peptide and gentamicin, to their respective target RNAs.

7.1. Materials and Methods

7.1.1. Buffers

Tris-potassium chloride (TK) buffer is composed of 50 mM Tris-HCl pH 7.4, 20mM KCl, 0.1%Triton X-100, and 0.5mM $MgCl_2$. Tris-borate-EDTA (TBE) buffer is

composed of 45 mM Tris-borate pH 8.0, and 1 mM EDTA. Tris-Potassium chloride-magnesium (TKM) buffer is composed of 50 mM Tris-HCl pH 7.4, 20mM KCl, 0.1%Triton X-100 and 5mM $MgCl_2$.

7.1.1. Gel retardation analysis

RNA oligonucleotides were purchased from Dharmacon, Inc, Lafayette, CO). 500 pmole of either a 5' fluorescein labeled oligonucleotide corresponding to the 16S rRNA A site (5'-GGCGUCACACCUUCGGGUGAAGUCGCC-3' (SEQ ID NO: 29); Moazed & Noller, 1987, Nature 327:389-394; Woodcock *et al.*, 1991, EMBO J. 10:3099-3103; Yoshizawa *et al.*, 1998, EMBO J. 17:6437-6448) or a 5' fluorescein labeled oligonucleotide corresponding to the HIV-1 TAR element TAR RNA (5'-GGCGUCACACCUUCGGGUGAAGUCGCC-3' (SEQ ID NO: 30); Huq *et al.*, 1999, Nucleic Acids Research. 27:1084-1093; Hwang *et al.*, 1999, Proc. Natl. Acad. Sci. USA 96:12997-13002) was 3' labeled with 5'-^{32}P cytidine 3', 5'-bis(phosphate) (NEN) and T4 RNA ligase (NEBiolabs) in 10% DMSO as per manufacturer's instructions. The labeled oligonucleotides were purified using G-25 Sephadex columns (Boehringer Mannheim). For Tat-TAR gel retardation reactions the method of Huq *et al.* (Nucleic Acids Research, 1999, 27:1084-1093) was utilized with TK buffer containing 0.5mM $MgCl_2$ and a 12-mer Tat peptide (YGRKKRRQRRRP (SEQ ID NO: 31); single letter amino acid code). For 16S rRNA-gentamicin reactions, the method of Huq *et al.* was used with TKM buffer. In 20 μl reaction volumes 50 pmoles of ^{32}P cytidine-labeled oligonucleotide and either gentamicin sulfate (Sigma) or the short Tat peptide (Tat_{47-58}) in TK or TKM buffer were heated at 90°C for 2 minutes and allow to cool to room temperature (approximately 24°C) over 2 hours. Then 10 μl of 30% glycerol was added to each reaction tube and the entire sample was loaded onto a TBE non-denaturing polyacrylamide gel and electrophoresed at 1200-1600 volt-hours at 4°C. The gel was exposed to an intensifying screen and radioactivity was quantitated using a Typhoon phosporimager (Molecular Dynamics).

7.2. Background

One method used to demonstrate small molecule interactions with natural occurring RNA structures such as ribosomes is by a method called chemical footprinting or toe printing (Moazed & Noller, 1987, Nature 327:389-394; Woodcock *et al.*, 1991, EMBO J. 10:3099-3103; Yoshizawa *et al.*, 1998, EMBO J. 17:6437-6448). Here the use of gel mobility shift assays to monitor RNA-small molecule interactions are described. This approach allows for rapid visualization of small molecule-RNA interactions based on the

difference between mobility of RNA alone versus RNA in a complex with a small molecule. To validate this approach, an RNA oligonucleotide corresponding to the well-characterized gentamicin binding site on the 16S rRNA (Moazed & Noller, 1987, Nature 327:389-394) and the equally well-characterized HIV-1 TAT protein binding site on the HIV-1 TAR element (Huq *et al.*, 1999, Nucleic Acids Res. 27: 1084-1093) were chosen. The purpose of these experiments is to lay the groundwork for the use of chromatographic techniques in a high throughput fashion, such as microcapillary electrophoresis, for drug discovery.

7.3. Results

A gel retardation assay was performed using the Tat_{47-58} peptide and the TAR RNA oligonucleotide. As shown in Figure 1, in the presence of the Tat peptide, a clear shift is visible when the products are separated on a 12% non-denaturing polyacrylamide gel. In the reaction that lacks peptide, only the free RNA is visible. These observations confirm previous reports made using other Tat peptides (Hamy *et al.*, 1997, Proc. Natl. Acad. Sci. USA 94:3548-3553; Huq *et al.*, 1999, Nucleic Acids Res. 27: 1084-1093).

Based on the results of Figure 1, it was hypothesized that RNA interactions with small organic molecules could also be visualized using this method. As shown in Figure 2, the addition of varying concentrations of gentamicin to an RNA oligonucleotide corresponding to the 16S rRNA A site produces a mobility shift. These results demonstrate that the binding of the small molecule gentamicin to an RNA oligonucleotide having a defined structure in solution can be monitored using this approach. In addition, as shown in Figure 2, a concentration as low as 10ng/ml gentamicin produces the mobility shift.

To determine whether lower concentrations of gentamicin would be sufficient to produce a gel shift, similar experiment was performed, as shown in Figure 2, except that the concentrations of gentamicin ranged from 100 ng/ml to 10 pg/ml. As shown in Figure 3, gel mobility shifts are produced when the gentamicin concentration is as low as 10 pg/ml. Further, the results shown in Figure 3 demonstrate that the shift is specific to the 16S rRNA oligonucleotide as the use of an unrelated oligonucleotide, corresponding to the HIV TAR RNA element, does not result in a gel mobility shift when incubated with 10 μg/ml gentamicin. In addition, if a concentration as low as 10 pg/ml gentamicin produces a gel mobility shift then it should be possible to detect changes to RNA structural motifs when small amounts of compound from a library of diverse compounds is screened in this fashion.

Further analysis of the gentamicin-RNA interaction indicates that the interaction is Mg- and temperature dependent. As shown in Figure 4, when $MgCl_2$ is not present (TK buffer), 1mg/ml of gentamicin must be added to the reaction to produce a gel shift.

Similarly, the temperature of the reaction when gentamicin is added is also important. When gentamicin is present in the reaction during the entire denaturation/renaturation cycle, that is, when gentamicin is added at 90C°C or 85°C, a gel shift is visualized (data not shown). In contrast, when gentamicin is added after the renaturation step has proceeded to 75°C, a mobility shift is not produced. These results are consistent with the notion that gentamicin may recognize and interact with an RNA structure formed early in the renaturation process.

8. EXAMPLE: IDENTIFICATION OF A DYE-LABELED TARGET RNA BOUND TO SMALL MOLECULAR WEIGHT COMPOUNDS BY CAPILLARY ELECTROPHORESIS

The results presented in this Example indicate that interactions between a peptide and its target RNA, such as the Tat peptide and TAR RNA, can be monitored by gel retardation assays in an automated capillary electrophoresis system.

8.1. Materials and Methods

8.1.1. Buffers

Tris-potassium chloride (TK) buffer is composed of 50 mM Tris-HCl pH 7.4, 20mM KCl, 0.1%Triton X-100, and 0.5mM $MgCl_2$. Tris-borate-EDTA (TBE) buffer is composed of 45 mM Tris-borate pH 8.0, and 1 mM EDTA. Tris-Potassium chloride-magnesium (TKM) buffer is composed of 50 mM Tris-HCl pH 7.4, 20mM KCl, 0.1%Triton X-100 and 5mM $MgCl_2$.

8.1.1. Gel Retardation Analysis Using Capillary Electrophoresis

RNA oligonucleotides were purchased from Dharmacon, Inc, Lafayette, CO). 500 pmole of a 5' fluorescein labeled oligonucleotide corresponding to the HIV-1 TAR element TAR RNA (5'-GGCGUCACACCUUCGGGUGAAGUCGCC-3' (SEQ ID NO: 30); Huq *et al.*, 1999, Nucleic Acids Research. 27:1084-1093; Hwang *et al.*, 1999, Proc. Natl. Acad. Sci. USA 96:12997-13002) was used. For Tat-TAR gel retardation reactions the method of Huq *et al.* (Nucleic Acids Research, 1999, 27:1084-1093) was

utilized with TK buffer containing 0.5mM $MgCl_2$ and a 12-mer Tat peptide (YGRKKRRQRRRP (SEQ ID NO: 31); single letter amino acid code). In 20 µl reaction volumes 50 pmoles of labeled oligonucleotide and the short Tat peptide (Tat_{47-58}) in TK or TKM buffer were heated at 90°C for 2 minutes and allow to cool to room temperature (approximately 24°C) over 2 hours. The reactions were loaded onto a SCE9610 automated capillary electrophoresis apparatus (SpectruMedix; State College, Pennsylvania).

8.2. Results

As presented in the previous Example in Section 7, interactions between a peptide and RNA can be monitored by gel retardation assays. It was hypothesized that interactions between a peptide and RNA could be monitored by gel retardation assays by an automated capillary electrophoresis system. To test this hypothesis, a gel retardation assay by an automated capillary electrophoresis system was performed using the Tat_{47-58} peptide and the TAR RNA oligonucleotide. As shown in Figure 5 using the capillary electrophoresis system, in the presence of the Tat peptide, a clear shift is visible upon the addition of increasing concentrations of Tat peptide. In the reaction that lacks peptide, only a peak corresponding to the free RNA is observed. These observations confirm previous reports made using other Tat peptides (Hamy *et al.*, 1997, Proc. Natl. Acad. Sci. USA 94:3548-3553; Huq *et al.*, 1999, Nucleic Acids Res. 27: 1084-1093).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

The invention can be illustrated by the following embodiments enumerated in the numbered paragraphs that follow:

1. A method for identifying a test compound that binds to a target RNA molecule, comprising the steps of (a) contacting a detectably labeled target RNA molecule with a library of test compounds under conditions that permit direct binding of the labeled target RNA to a member of the library of test compounds so that a detectably labeled target RNA:test compound complex is formed; (b) separating the detectably labeled target RNA:test compound complex formed in step(a) from uncomplexed target RNA molecules and test compounds; and (c) determining a structure of the test compound bound to the RNA in the RNA:test compound complex.

2. The method of paragraph 1 in which the target RNA molecule contains an HIV TAR element, internal ribosome entry site, "slippery site", instability element, or adenylate uridylate-rich element.

3. The method of paragraph 1 in which the RNA molecule is an element derived from the mRNA for tumor necrosis factor alpha ("TNF-α"), granulocyte-macrophage colony stimulating factor ("GM-CSF"), interleukin 2 ("IL-2"), interleukin 6 ("IL-6"), vascular endothelial growth factor ("VEGF"), human immunodeficiency virus I ("HIV-1"), hepatitis C virus ("HCV" - genotypes 1a & 1b), ribonuclease P RNA ("RNaseP"), X-linked inhibitor of apoptosis protein ("XIAP"), or survivin.

4. The method of paragraph 1 in which the detectably labeled RNA is labeled with a fluorescent dye, phosphorescent dye, ultraviolet dye, infrared dye, visible dye, radiolabel, enzyme, spectroscopic colorimetric label, affinity tag, or nanoparticle.

5. The method of paragraph 1 in which the test compound is selected from a combinatorial library comprising peptoids; random bio-oligomers; diversomers such as hydantoins, benzodiazepines and dipeptides; vinylogous polypeptides; nonpeptidal peptidomimetics; oligocarbamates; peptidyl phosphonates; peptide nucleic acid libraries; antibody libraries; carbohydrate libraries; and small organic molecule libraries, including but not limited to, libraries of benzodiazepines, isoprenoids, thiazolidinones, metathiazanones, pyrrolidines, morpholino compounds, or diazepindiones.

6. The method of paragraph 1 in which screening a library of test compounds comprises contacting the test compound with the target nucleic acid in the presence of an aqueous solution, the aqueous solution comprising a buffer and a combination of salts, preferably approximating or mimicking physiologic conditions.

7. The method of paragraph 6 in which the aqueous solution optionally further comprises non-specific nucleic acids comprising DNA, yeast tRNA, salmon sperm DNA, homoribopolymers, and nonspecific RNAs.

8. The method of paragraph 6 in which the aqueous solution further comprises a buffer, a combination of salts, and optionally, a detergent or a surfactant. In another embodiment, the aqueous solution further comprises a combination of salts, from about 0 mM to about 100 mM KCl, from about 0 mM to about 1 M NaCl, and from about 0 mM to about 200 mM $MgCl_2$. In a preferred embodiment, the combination of salts is about 100 mM KCl, 500 mM NaCl, and 10 mM $MgCl_2$. In another embodiment, the solution optionally comprises from about 0.01% to about 0.5% (w/v) of a detergent or a surfactant.

9. Any method that detects an altered physical property of a target nucleic acid complexed to a test compound from the unbound target nucleic acid may be used for separation of the complexed and non-complexed target nucleic acids in the method of paragraph 1. In a preferred embodiment, electrophoresis is used for separation of the complexed and non-complexed target nucleic acids. In a preferred embodiment, the electrophoresis is capillary electrophoresis. In other embodiments, fluorescence spectroscopy, surface plasmon resonance, mass spectrometry, scintillation, proximity assay, structure-activity relationships ("SAR") by NMR spectroscopy, size exclusion chromatography, affinity chromatography, and nanoparticle aggregation are used for the separation of the complexed and non-complexed target nucleic acids.

10. The structure of the test compound of the RNA:test compound complex of paragraph 1 is determined, in part, by the type of library of test compounds. In a preferred embodiment wherein the combinatorial libraries are small organic molecule libraries, mass spectroscopy, NMR, or vibration spectroscopy are used to determine the structure of the test compounds.

WHAT IS CLAIMED IS:

1. A method for identifying a test compound that binds to a target RNA molecule, comprising the steps of:

(a) contacting a detectably labeled target RNA molecule with a library of test compounds under conditions that permit direct binding of the labeled target RNA to a member of the library of test compounds so that a detectably labeled target RNA:test compound complex is formed;

(b) separating the detectably labeled target RNA:test compound complex formed in step(a) from uncomplexed target RNA molecules and test compounds by capillary gel electrophoresis; and

(c) determining a structure of the test compound bound to the RNA in the RNA:test compound complex by mass spectroscopy.

AMENDED CLAIMS

[received by the International Bureau on 17 September 2002 (17.09.02);
Claims 1 to 10 replaced by new claims 1 to 19. (3 sheets)]

1. A method for identifying a test compound that binds to a target RNA molecule, comprising the steps of:

(a) contacting a detectably labeled target RNA molecule with a library of test compounds under conditions that permit direct binding of the labeled target RNA to a member of the library of test compounds so that a detectably labeled target RNA:test compound complex is formed;

(b) separating the detectably labeled target RNA:test compound complex formed in step (a) from uncomplexed target RNA molecules and test compounds; and

(c) determining a structure of the test compound bound to the RNA in the RNA:test compound complex.

2. The method of claim 1 in which the target RNA molecule contains an HIV TAR element, internal ribosome entry site, "slippery site", instability element, or adenylate uridylate-rich element.

3. The method of claim 1 in which the RNA molecule is an element derived from the mRNA for tumor necrosis factor alpha ("TNF-α"), granulocyte-macrophage colony stimulating factor ("GM-CSF"), interleukin 2 ("IL-2"), interleukin 6 ("IL-6"), vascular endothelial growth factor ("VEGF"), human immunodeficiency virus I ("HIV-1"), hepatitis C virus ("HCV" - genotypes 1a & 1b), ribonuclease P RNA ("RNaseP"), X-linked inhibitor of apoptosis protein ("XIAP"), or survivin.

4. The method of claim 1 in which the detectably labeled RNA is labeled with a fluorescent dye, phosphorescent dye, ultraviolet dye, infrared dye, visible dye, radiolabel, enzyme, spectroscopic colorimetric label, affinity tag, or nanoparticle.

5. The method of claim 1 in which the test compound is selected from a combinatorial library comprising peptoids; random bio-oligomers; diversomers such as hydantoins, benzodiazepines and dipeptides; vinylogous polypeptides; nonpeptidal

peptidomimetics; oligocarbamates; peptidyl phosphonates; peptide nucleic acid libraries; antibody libraries; carbohydrate libraries; or small organic molecule libraries.

6. The method of claim 5 in which the small organic molecule libraries are libraries of benzodiazepines, isoprenoids, thiazolidinones, metathiazanones, pyrrolidines, morpholino compounds, or diazepindiones.

7. The method of claim 1 in which screening a library of test compounds comprises contacting the test compound with the target nucleic acid in the presence of an aqueous solution wherein the aqueous solution comprises a buffer and a combination of salts.

8. The method of claim 7 wherein the aqueous solution approximates or mimics physiologic conditions.

9. The method of claim 7 in which the aqueous solution optionally further comprises non-specific nucleic acids comprising DNA, yeast tRNA, salmon sperm DNA, homoribopolymers, and nonspecific RNAs.

10. The method of claim 7 in which the aqueous solution further comprises a buffer, a combination of salts, and optionally, a detergent or a surfactant.

11. The method of claim 10 in which the aqueous solution further comprises a combination of salts, from about 0 mM to about 100 mM KCl, from about 0 mM to about 1 M NaCl, and from about 0 mM to about 200 mM $MgCl_2$.

13 The method of claim 11 wherein the combination of salts is about 100 mM KCl, 500 mM NaCl, and 10 mM $MgCl_2$.

14. The method of claim 10 wherein the solution optionally comprises from about 0.01% to about 0.5% (w/v) of a detergent or a surfactant.

15. The method of claim 1 in which separating the detectably labeled target RNA:test compound complex formed in step (a) from uncomplexed target RNA and test compounds is by electrophoresis.

16. The method of claim 15 in which the electrophoresis is capillary electrophoresis.

17. The method of claim 1 in which separating the detectably labeled target RNA:test compound complex formed in step (a) from uncomplexed target RNA and test compounds is by fluorescence spectroscopy, surface plasmon resonance, mass spectrometry, scintillation, proximity assay, structure-activity relationships ("SAR") by NMR spectroscopy, size exclusion chromatography, affinity chromatography, or nanoparticle aggregation.

18. The method of claim 1 in which the library of test compounds are small organic molecule libraries.

19. The method of claim 18 in which the structure of the test compound is determined by mass spectroscopy, NMR, or vibration spectroscopy.

Figure 1

Sheet 1/5

Attorney Docket No. 10589-007

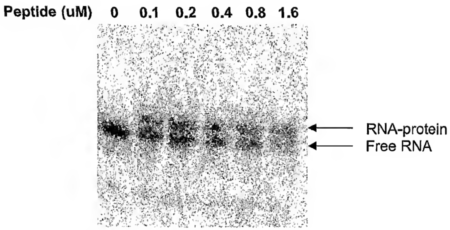

Figure 2

Sheet 2/5

Attorney Docket No. 10589-007

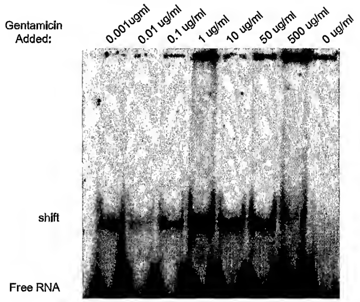

Figure 3

Sheet 3/5

Attorney Docket No. 10589-007

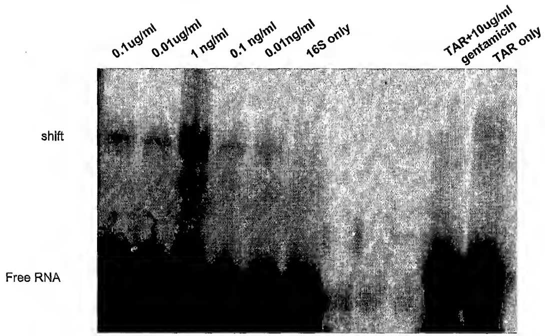

Figure 4

Sheet 4/5

Attorney Docket No. 10589-007

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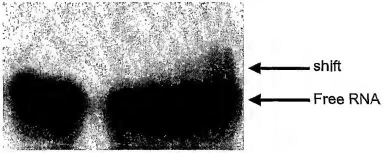
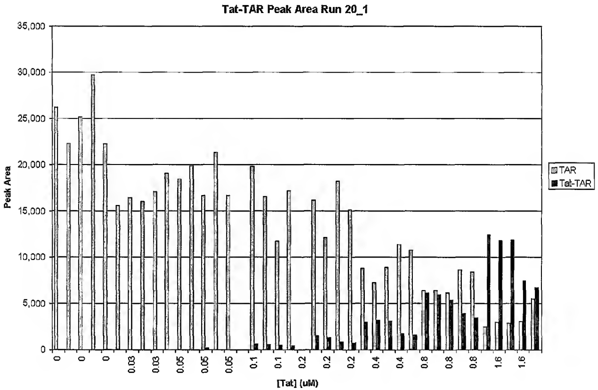


Figure 5

Sheet 5/5

Attorney Docket No. 10589-007



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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/11757

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12Q 1/68; C07H 21/02; G01N 27/26
US CL : 435/6; 536/23.1; 204/451
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6; 536/23.1; 204/451

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN, EAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6329146 B1 (Crooke et al) 11 December 2001 (11.12.2001), column 40, example 11.	1
Y	US 5,807,682 A (Grossman et al) 15 September 1998 (15.09.1998), column 19, lines 2-18.	1
Y	US 6,355,428 B1 (Schroth et al) 12 March 2002 (12.03.2002), column 8, lines 64-67.	1
Y	US 6,320,040 B1 (Cook et al) 20 November 2001 (20.11.2001), column 11, lines 14-22	1
Y	US 6,391,542 B1 (Anderson et al) 12 May 2002 (12.05.2002), column 36, example 18.	1

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 22 June 2002 (22.06.2002)	Date of mailing of the international search report 18 JUL 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Jyothsna Venkat JYOTHSNA VENKAT PH.D SUPERVISORY PATENT EXAMINER TECHNOLOGY CENTER 1600 Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet) (July 1998)